Second edition

Viral Pathogenesis and Immunity

Neal Nathanson

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VISIT…

Viral Pathogenesis and Immunity

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Preface

Infectious disease is one of the few genuine adventures left in the world. The dragons are all dead and the lance grows rusty in the chimney corner... About the only sporting proposition that remains unimpaired by the relentless domestication of a once free-living human species is the war against those ferocious little fellow creatures, which lurk in the dark corners and stalk us in the bodies of rats, mice and all kinds of domestic animals; which fly and crawl with the insects, and waylay us in our food and drink and even in our love.

Hans Zinsser, Rats, Lice and History, 1935

To wrest from nature the secrets which have perplexed philosophers in all ages, to track to their sources the cause of disease . . . these are our ambitions.

William Osler

This book is the direct offspring of *Viral Pathogenesis*, published in 1997. Having read several drafts of all the chapters in *Viral Pathogenesis*, it was clear that the large book contained a wealth of information, but that it was unsuited for readers who desired an introduction to the topic. From that observation sprang the plan for a short version that could be used as an introductory text or for self-education.

An introductory text clearly benefits from the coherence provided by a single author, but suffers from the finite expertise of any single researcher. Therefore, a compromise was devised, in which each chapter was co-authored by an expert in the specific area under consideration. This strategy was facilitated by the successful collaborations that had been developed during the preparation of *Viral Pathogenesis*. The text includes references published through to June, 2006.

I would like to acknowledge the contributions of the co-authors. Their advice and expertise has been essential to the planning and execution of this undertaking and it has been a continual pleasure to work with them. They have provided extremely cogent suggestions that have given the book an enhanced level of authority that could not otherwise have been achieved.

Lisa Tickner and the staff at Academic Press, London, have been an ongoing source of support both because of their enthusiasm for this book and their highly professional expertise in all phases of the project. Wendy Jackelow provided the outstanding illustrations rendered from a wide variety of often primitive sketches.

Neal Nathanson
Philadelphia

Introduction

Viral Pathogenesis and Immunity treats all aspects of infection of the animal host, including the sequence of events from entry to shedding, the clearance or persistence of the virus, the immune response of the host and the subsequent occurrence of disease. Particular attention is focused on mechanisms that explain the complex interaction between parasite and host.

This book is designed to provide an introductory overview of viral pathogenesis in a format which will be easy for the reader to absorb without recourse to additional information. Principles are emphasized and no attempt is made to provide a virus-by-virus or disease-by-disease compendium, since these are already available in texts of microbiology and infectious diseases. Examples are given to illustrate the principles but they are representative not encyclopedic. By keeping to essentials, it is hoped to provide a coherent introduction in a brief compass, leaving the reader to acquire more detailed information from well-documented comprehensive texts.

It is assumed that the reader knows the fundamentals of virology, including the structure of viruses, the organization of their genomes, the basic steps in viral replication, assembly and release. In addition, a basic background in cell biology, immunology and pathology will be useful. Students who have taken an introductory course in microbiology will have acquired this background and should be well equipped to use this book. For those who wish to review these essentials, many excellent texts are available and some outstanding ones are noted below. In addition, at the end of each chapter some selected references are provided for readers who wish to delve more deeply into the subject matter or to read a few of the classical original contributions to the field.

Viral Pathogenesis and Immunity is divided into four parts. Part I, *Essentials of viral pathogenesis*, acquaints the reader with the sequential events in viral infections, the dissemination of virus in the host and the variety of cellular responses to infection. Part II, *Host responses to viral infection*, describes the non-specific and specific immune responses to infection, including the immunopathological and immunosuppressive consequences of infection. Part III, *Virus–host interactions*, deals with virus virulence, virus persistence, virus-induced oncogenesis and the determinants of host susceptibility. Part IV, *Ecology and control of viral infections*, applies the principles of pathogenesis to emergence, treatment and prevention of infection.

This organization permits readers to select those subjects of particular interest to them, depending upon their background, goals and available time. Thus, it would be possible to base an abbreviated introduction to the subject upon Parts I and III alone, particularly for readers with some background in immunology.

BACKGROUND READING

Abbas AK, Lichtman AH. *Cellular and Molecular Immunology*, 5th edn, Saunders, Philadelphia, 2003. *Selected chapters will provide a thorough and clear introduction for readers who have not taken a course in immunology.*

Brooks GF, Butel JS, Morse SA. *Jawetz, Melnick and Adelberg's Medical Microbiology*, 23rd edn, Lange Medical Books/McGraw-Hill, New York, 2004. *Basic chapters on properties of viruses and on immunology will provide sufficient background for readers who have not taken a course in microbiology or immunology.*

Janeway CA Jr, Travers P, Walpot M, Shlomchik MJ. *Immunobiology*, 6th edn, Garland Science, New York, 2005. *An alternative introductory immunology text.*

FURTHER READING (GENERAL REFERENCES)

Flint SJ, Enquist LW, Racaniello VR, Skalka AM. *Principles of virology*, 2nd edn, ASM Press, Washington, DC, 2004. *An excellent detailed virology text.*

Knipe DM, Howley PM (eds). *Fields' Virology*, 5th edn, Lippincott Williams and Wilkins, Philadelphia, 2007. *This exhaustive reference provides definitive information about basic virology and about individual viruses and virus families.*

Richman DD, Whitley RJ, Hayden FG (eds). *Clinical virology*, 2nd edn, ASM Press, Washington, DC, 2002. *This authoritative reference describes viral diseases, supplemented with much basic background information.*

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PART I

Essentials of Viral Pathogenesis

Chapter 1

Historical Roots

Neal Nathanson and Frederick A. Murphy

CHAPTER CONTENTS

HISTORY OF INFECTIOUS DISEASES AND MICROBIOLOGY

EARLY STUDIES OF PATHOGENESIS: 1900–1950

THE CLASSIC ERA: 1950–1975

THE ERA OF MOLECULAR BIOLOGY: 1975–2000

PATHOGENESIS IN THE NEW MILLENNIUM

FURTHER READING

HISTORY OF INFECTIOUS DISEASES AND MICROBIOLOGY

The history of viral pathogenesis is intertwined with the history of medicine. Ancient physicians recorded clinical illnesses, understanding that classification of diseases was a prerequisite for prescribing remedies, although treatments were often of questionable value. Viral diseases that were clearly recognized in ancient times were those, such as poliomyelitis, that produced distinctive or unique signs and symptoms. In a few instances, for example rabies, where illness often followed upon the bite of a rabid dog or wolf, even the transmissible nature of the illness was clearly understood (Figure 1.1).

From the time of early civilizations relatively little progress was made until the development of modern science that began in the Renaissance, sparked by the prolific genius of Leonardo da Vinci (1452–1519). Girolamo Fracastoro, writing in the 16th century, proposed a theory of contagions caused by 'small imperceptible particles' that were transmitted either by contact, by fomites or over distances. Fracastoro's theoretical treatise, though based on speculation, was a remarkably prescient vision that paved the way for the discovery of microbial organisms. The actual beginnings of microbiology are dated by some historians to the late 17th century when Antony van Leeuwenhoek described bacteria and other unicellular organisms seen through the microscopes that he built himself. Microorganisms could readily be observed in infusions or in putrefying materials and one controversial question was whether they arose by spontaneous generation. In the late 18th century, Lazaro Spallanzani devised some simple but telling experiments showing that organisms in a flask could be killed by heating or boiling and did not reappear if the flask had been sealed to preclude reseeding from the air.

Nevertheless, understanding of the nature of infection was relatively primitive in the late 18th century. For instance, the yellow fever epidemic in Philadelphia in 1793 engaged the best medical minds of 18th century America, including Benjamin Rush, generally acknowledged to be the leading physician of the colonies. Rush hypothesized that the disease arose from some effluvium deposited on the docks by ships recently arrived from the Caribbean, apparently not focusing on the human cases of yellow fever imported by the same ships. Furthermore, he prescribed a regimen of frequent 'cupping' (therapeutic bleeding) that only served to debilitate the

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FIGURE 1.1 Rabid dog biting a man. Arabic painting by Abdallah ibn al-Fadl, Baghdad school, 1224. Courtesy of the Freer Gallery of Art, Washington, DC. After Baer G (ed.), *The natural history of rabies*, 2nd edn, CRC Press, Boca Raton, 1991.

mortally affected sufferers. It remained for Pasteur, in the mid-19th century, to develop the concept that each communicable disease was associated with its own unique causal agent (see below).

Viral epidemics, in which most clinical cases were due to a single organism, indicated the transmissible nature of infections and demonstrated stages in the evolution of the infectious process. One instance is the epidemic of measles that occurred in the Faroe Islands in the north Atlantic in 1846, recorded by Peter Panum, a young Danish physician. The disease was introduced by a cabinetmaker from Copenhagen who arrived on 28 March and developed measles in early April. Between April and October, over 6000 cases occurred among the population of almost 7900, with over 170 deaths (Table 1.1).

From a number of simple clinical observations, Panum drew several important inferences. First, the disease was clearly transmitted from person to person by direct contact and spread in this fashion to overtake almost the whole population. This strongly suggested that measles was caused by a specific agent, contradicting the vague miasma theory of febrile diseases that had been popular for centuries. Second, it appeared that most cases in the epidemic exhibited consistent signs and symptoms, such as the typical rash, suggesting that each transmissible disease might be due to a distinct agent. Third, the interval from exposure to onset was about two weeks and the patient was contagious at the onset of illness, indicating a stereotyped natural history. For measles, this included a silent incubation period, followed by a febrile rash with virus shedding. Finally, the outcome of illness was influenced by age, with highest mortality among infants and the very elderly, one of the first documented instances of variable host responses to a single infectious agent.

In the 19th century, the theory of spontaneous generation was definitively refuted by a number of workers,

Age group	Annual mortality per 100		
	1835–1845	1846	Excess in 1846
<1	10.8	30	19.2
1–9	0.5	0.5	—
10–19	0.5	0.5	—
20–29	0.5	0.7	0.2
30–39	0.8	2.1	1.3
40–49	1.1	2.7	1.6
50–59	0.9	4.4	3.5
60–69	2	7.7	5.7
70–79	6.5	13.1	6.6
80–100	16.8	26	9.2

TABLE 1.1 Age-specific differences in mortality from measles. Data from the measles epidemic in the Faroe Islands, 1846, compared with average mortality for 1835–1845. The excess mortality for 1846 provides a crude estimate of measles-specific mortality during the epidemic, which involved at least 75% of the population
Data from Panum PL. Observations made during the epidemic of measles on the Faroe Islands in the year 1846, American Public Health Association, New York: 1940, with permission.

SIDEBAR 1.1

The Henle-Koch postulates, as originally framed (1840 and 1890)

- The incriminated agent can be cultured from lesions of the disease
- The incriminated agent does not occur as a fortuitous and non-pathogenic contaminant in individuals who are healthy or have other diseases
- The agent can be grown in pure culture
- The agent reproduces the disease when introduced into an appropriate host
- The agent can be recultured from the diseased host

During the last century, revised and expanded versions of these postulates have been developed because experience has indicated that there are numerous exceptions to the guidelines as originally framed. Certain infectious agents, such as several hepatitis viruses, cannot be 'cultured' and, in some instances, there is no non-human host in which the disease can be reproduced. Also, new methods in virology, molecular genetics, immunology, epidemiology and biostatistics provide many more ways to confirm a causal relationship. Finally, as emphasized by Evans, the postulated relationship between organism and disease 'must make biological sense'. (See Evans AS. Causation and disease: a chronological journey. *American Journal of Epidemiology* 1978, 108: 249–258.)

particularly Schwann in 1837 and Cagniard-Latour in 1838. In 1857, Pasteur found that different fermentations were associated with different microbial agents, providing further evidence against spontaneous generation and setting the stage for the idea that each infection was caused by a specific agent. In 1850, Semmelweis inferred that physicians were spreading childbed fever, a streptococcal infection, by failing to wash their hands and, in 1867, Lister showed that carbolic acid applied as an antiseptic could reduce postoperative infections; these advances strengthened the belief in the microbial origin of infection and contributed practical applications of the concept.

In the second half of the 19th century, rapid advances were made. One after another, the causal bacteria responsible for important infections were defined, beginning with isolation of the anthrax bacillus from the blood of infected animals by Davaine in 1865 and its transmission to mice by Koch in 1877. In 1881, Koch was able to grow bacteria on solid media, facilitating the isolation of pure cultures of single organisms. In 1884, Koch, drawing on the ideas enunciated in 1840 by his teacher Jacob Henle, conceptualized the relationship between individual infectious agents and specific diseases as a series of axioms commonly known as the Henle-Koch postulates (Sidebar 1.1).

Viruses were discovered as a direct outgrowth of these studies of bacterial agents. Between 1886 and 1892, Mayer and Beijerinck, working at the Agricultural Experimental Station in Wageningen, Holland, and Ivanovsky, working independently in Russia, demonstrated that mosaic disease of tobacco could be transmitted from plant to plant by extracts of infected vegetation. Furthermore, no bacterial agent could be grown from these extracts and the infectivity could pass through Chamberland filters, i.e. porcelain filters with a pore size of 100–500 nm that excluded most bacteria.

We now consider these observations to represent the discovery of the first recognized virus, tobacco mosaic virus. However, at the time, there was a controversy whether the causal agent was in fact a bacterium capable of passing through the filters and incapable of growing on the medium used, or the first representative of a new class of agents. Beijerinck showed that the infectious agent multiplied in plant tissues but not in the sap and championed the latter view, naming the class of causal agents 'contagium vivum fluidum' or 'contagious living fluid'. Shortly after these studies, and informed by them, the first animal viruses were identified: foot-and-mouth virus, a picornavirus, and yellow fever virus, a flavivirus.

Foot-and-mouth disease is a highly contagious, sometimes fatal, vesicular disease of cattle and swine that was a serious problem for farmers in Germany. Loeffler and Froesch from the Berlin Institute for Infectious Diseases, one of the foremost institutions for infectious disease research in the late 19th century, were commissioned to study the problem. Friedrich Loeffler, an early collaborator of Koch, had been trained as a bacteriologist, which prepared him to consider whether a newly recognized agent had the characteristics of bacteria.

The report of their investigation of foot-and-mouth disease is astoundingly modern. With impeccable logic, the investigators focused on fluids obtained by puncturing

alcohol-sterilized early vesicles, the one source where the infectious agent could be obtained free of contaminating skin bacteria. By rigorous techniques they excluded bacteria, although they scrupulously noted that they could not rule out bacteria incapable of growing on the media used and invisible in their microscopes.

Using filtration (controlled by samples to which known bacterial strains had been added to eliminate potentially undetected bacteria), they showed that the causal agent was present in high titer in filtered lymph from infected animals. Two explanations remained: either they were dealing with a toxin or with a sub-bacterial infectious agent. Careful calculations of the cumulative dilutions produced by serial passage indicated that either the toxin was even more virulent than tetanus toxin, the most potent bacterial toxin then known, or the disease was caused by a replicating agent. In the latter case, the organism was smaller than known bacteria and incapable of growth on bacterial media. In conclusion, the authors recognized that the foot-and-mouth disease agent might be the prototype of a new class of agents and they nominated smallpox and vaccinia as potential members of this class. The combination of rigorous thinking, meticulous execution and far-reaching insights marks their report as truly unique, a paper that is astonishing to read more than 100 years after its publication.

During the 17th, 18th and 19th centuries, urban yellow fever was endemic in the major cities of South America and the Caribbean and intermittently epidemic in many of the major ports of North America. In 1900, the United States Army sent Major Walter Reed to Cuba to head a commission to study yellow fever, which was causing devastating morbidity and mortality in troops stationed in the Caribbean theater. The commission arrived during a severe outbreak of disease and set to work to identify the causal agent. After eliminating a bacterial candidate, *Bacillus icteroides*, as a secondary invader, they decided to test the hypothesis that the agent was transmitted by mosquitoes, which had been proposed 20 years before by Carlos Finlay, a Cuban physician.

They devised a trial in which volunteer soldiers were divided into two groups: one group used bedding previously occupied by soldiers with acute yellow fever, but were housed in barracks that were screened to exclude mosquitoes, while the other group occupied clean barracks that were unscreened. Only troops in the unscreened barracks developed the disease. Using colonized *Aedes aegypti* mosquitoes obtained from Dr Finlay, Reed and his colleagues, particularly James Carroll, were able to transmit the disease by mosquitoes that had fed on acutely ill patients and then, about two weeks later, on human volunteers. Furthermore, they demonstrated that blood obtained from acutely ill patients would transmit the disease to volunteers. At the suggestion of William Welch, the famous pathologist from the Johns Hopkins University, who was aware of the work of Loeffler and Frosch, they injected three volunteers with serum from patients in the early phases of yellow fever, which had been diluted and passed through a Berkfeld bacteria-excluding filter; two of the volunteers developed the disease. This was the first demonstration that an infectious disease of humans was caused by a virus (Sidebar 1.2).

EARLY STUDIES OF VIRAL PATHOGENESIS: 1900–1950

Virology was severely constrained in the first half of the 20th century by several technical limitations, the most important of which was the lack of a cell culture system for growing and titrating viruses. In the absence of methods for detecting viruses in tissues, observations of experimental infections were limited to clinical signs and pathological lesions that represented the endstage of disease. In spite of these adverse circumstances, extensive studies were undertaken of a few infections, such as poliomyelitis and Rous sarcoma.

Poliovirus was first isolated in 1908 by Landsteiner and Popper, who transmitted the infectious agent to monkeys by injection of a homogenate of the spinal cord from an acutely fatal human case. It was observed early on that the infection could not be transmitted to laboratory rodents; therefore, virus stocks were prepared by monkey-to-monkey passage, using the intracerebral route of inoculation. Investigators did not appreciate that this procedure neuroadapted the virus, changing its biological properties. Many experiments were performed using the MV (mixed virus) stock of poliovirus, later shown to be a type 2 strain that was an obligatory neurotropic virus. With the MV strain, the only 'natural' route by which rhesus monkeys could be infected was by intranasal instillation; it was later shown that the MV strain spread up the olfactory nerve to the brainstem and thence to the spinal cord to destroy the lower motor neurons resulting in flaccid paralysis.

These experiments led to the conviction that all polioviruses were neurotropic (viruses that mainly replicate in neural tissues), a scheme of pathogenesis that was widely accepted when it was summarized by Simon

SIDEBAR 1.2

Origin of the word 'virus'

The word *virus* is derived from the Latin for 'poison' and was traditionally used for the cause of any transmissible disease. With the discovery of agents that could pass bacteria-retaining filters, the term 'filterable virus' was introduced and this was later shortened to 'virus'. Pioneering virologists crafted biological definitions emphasizing that viruses were obligate intracellular parasites which, in their extracellular vegetative phase, formed particles smaller than bacteria (virus particles or virions range in size from ~15 to 300 nm) and that these virions could, in some cases, be crystallized like chemical compounds. Subsequently, modern genetic and biochemical definitions of viruses were introduced, which emphasized that viral genomes consisted of RNA or DNA, that encoded structural proteins that were incorporated into the virus particle and non-structural proteins that were essential for replication, transcription, translation and processing of the viral genome. Probably the most succinct description is that of Peter Medawar, 'bad news wrapped in protein'.

Flexner in 1931. This view of the pathogenesis of poliomyelitis led, in the summer of 1936, to a trial employing zinc sulfate as an astringent nasal spray; although the treatment produced some cases of anosmia, it did not prevent poliomyelitis. The failure of this trial stimulated a re-examination of the pathogenesis of poliomyelitis, that was radically revised only after the introduction in 1949 of cell culture methods by Enders, which permitted the isolation and propagation of virus strains that retained the properties of wild virus during laboratory passage. In turn, these discoveries led to the development of inactivated poliovirus vaccine (Sidebar 1.3).

SIDEBAR 1.3

The development of inactivated poliovirus vaccine

'In 1945, Professor Burnet of Melbourne (Macfarlane Burnet, subsequently to receive a Nobel prize) wrote: "While I was in America recently I had good opportunity to meet with most of the men actively engaged on research in poliomyelitis. . . The part played by acquired immunity to poliomyelitis is still completely uncertain, and the practical problem of preventing infantile paralysis has not been solved. It is even doubtful whether it ever will be solved." Most of us doing research on poliomyelitis in 1945 were mainly motivated by curiosity, rather than by the hope of a practical solution in our lifetime.' Yet, in 1954, less than 10 years later, a successful trial of inactivated poliovirus ('Salk') vaccine was underway. What happened in the interval illustrates the importance of understanding pathogenesis for the development of practical methods for the control of viral diseases.

The chain of discoveries is readily followed. First, in 1949, Enders, Weller and Robbins showed that it was possible to make cell cultures from a number of human tissues and that some of these cells would support the replication of poliovirus with a very obvious cytopathic effect. For the first time, it was readily possible to isolate wild strains of poliovirus and show that the virus was excreted in the feces of patients undergoing acute poliomyelitis, strongly suggesting that the causal agent entered its host by ingestion and replicated in the gastrointestinal tract, a view that had been espoused by Swedish workers in the early 20th century but had been discarded by later investigators. Fresh field isolates grown in cell cultures were now available for experimental study in primates and monkeys could be infected by feeding these isolates. Furthermore, and critically, it was now possible to show that the virus produced a plasma viremia and travelled through the blood to reach the spinal cord where it attacked anterior horn cells to cause flaccid paralysis, its dreaded hallmark.

Tissue culture methods permitted the development of a simple and rapid method for the measurement of neutralizing antibodies and a combination of studies, using cell culture assays and monkey challenges, showed that all poliovirus isolates could be grouped into three types, with neutralization and cross-protection within each type but not between types. Pooled sera from convalescent primates or from normal humans (gamma globulin) had substantial neutralizing titers and, administered prior to challenge with wildtype poliovirus, were shown to protect monkeys and chimpanzees against paralysis. It now remained to develop a vaccine to induce neutralizing antibodies and this was accomplished by Salk and his colleagues in the early 1950s using formalin to inactivate poliovirus purified from mass produced batches of virus. His studies showed that infectivity could be ablated while antigenicity was maintained, so as to induce the desired antibody response. Furthermore, a multivalent vaccine could be made, containing viruses of each antigenic type. In retrospect, understanding the role of viremia in infection, simple though it was, provided the logical basis for identifying neutralizing antibody as the immune correlate of protection, which established a rational basis for development of the vaccine. This account (and the quotes) has been freely adapted from Bodian D. Poliomyelitis and the sources of useful knowledge. *Johns Hopkins Medical Journal*, 1976, 138: 130–136 and Nathanson N. David Bodian's contribution to the development of poliovirus vaccine. *American Journal of Epidemiology*, 2005, 161: 207–212, with permission.

Peyton Rous' identification of the avian sarcoma virus that still bears his name, is a remarkable example of pioneering work that was recognized by a Nobel prize in 1966. Experimental transplantation of tumors was first accomplished at the beginning of the 20th century by the immunologist, Paul Ehrlich, who successfully adapted several mouse mammary carcinomas so that they could be transplanted to many strains of mice. These experiments demonstrated that transplantation was facilitated by the use of newborn or very young animals, by the intraperitoneal route of transfer and by the use of cell suspensions rather than solid tumor masses.

Based on these observations, Rous began his studies of the sarcomas of domestic chickens. In the Plymouth Rock breed, a partially inbred line of chickens, tumors could be transferred by subcutaneous inoculation and became more aggressive on serial passage. Rous made the seminal observation that the tumors could be passed by cell-free extracts, which were still active after filtration through a bacteria-retaining filter. Furthermore, chickens could be immunized to resist tumor transplantation and it was possible to differentiate immunity experimentally against whole tumor cells from immunity against the filtrable tumor-producing agent. These seminal studies were published between 1910 and 1913 but, due to the technical limitations of experimental virology, little additional progress was made over the next 40 years. With the advent of new methods in cell biology and molecular genetics, between 1955 and 1980, Rous sarcoma virus became a prototype system for the discovery of reverse transcriptase, the identification of retroviruses and the discovery of oncogenes.

THE CLASSIC ERA: 1950–1975

The study that ushered in the quantitative era of viral pathogenesis was Frank Fenner's classical investigation of mousepox (also called infectious ectromelia). Mousepox, a smallpox-like infection of mice, was shown to be caused by a transmissible virus in 1931 and, in 1937, Burnet reported that the agent could be quantitatively assayed on the chorioallantoic membrane of embryonated chicken's eggs; antibody could also be titrated by this method or by inhibition of its ability to agglutinate red blood cells under controlled conditions. These technical advances laid the way for Fenner to describe the sequential course of experimental infection, from entry by intradermal inoculation, to viremia, to spread to the liver and skin and transmission by virus-contaminated skin shed from cutaneous pox. This information was summarized in 1948 in a classic diagram that conveys the dynamics of the infection (Figure 1.2).

In the classical era, the most significant of the early breakthroughs was the development of methods for the culture of primary and continuous lines of mammalian cells. In 1952, exploiting cell cultures, Dulbecco demonstrated that viruses could be assayed by the plaque method, which was derived from the colony counts used to titrate bacteria and the plaque assays used for quantitating bacterial viruses (also called bacteriophages). A variant of this approach was used for tumor viruses that could be assayed in culture for their ability to produce foci of transformed cells.

A second significant advance was pioneered by Coons who, in 1953, introduced a procedure for the identification of viral antigens in cells. This made it possible to localize an agent to specific tissues and cell types in the infected host and to observe its progressive spread during the course of infection. This method depended on the development of techniques for the chemical labeling of antibody molecules with fluorescent 'tags' so that the antibody could be visualized microscopically using an ultraviolet light source. Beginning around 1955, electron microscopy was introduced to permit morphological observations at a subcellular level so that certain steps in the intracellular replication of viruses could be visualized together with the pathological consequences in individual cells. These histological methods complemented the quantitative assays of viral titers in tissue homogenates and body fluids.

A third important advance was the introduction of techniques for measurement of the immune response to viral infections. In the 1950s, methods were established for measurement of antiviral antibody, using neutralization, complement fixation, hemagglutination inhibition and other assays. Primitive assays of cellular immunity, such as delayed hypersensitivity following intradermal injection of antigen, were introduced in the 1940s, but it was not until the 1970s that more quantitative methods became available with the application of in vitro assays for cytolytic T lymphocytes by Doherty, Zinkernagel and others.

Using these methods, and following Fenner's example, classic studies were conducted of a number of viral infections. Noteworthy examples are studies of poliomyelitis by Bodian, Howe, Morgan and others (1940–1960), of arboviruses and ectromelia by Mims (1950s), of arboviruses and rabies by Johnson (1960s), of rabies by Murphy, Baer and others (1970s) and of lymphocytic choriomeningitis (Sidebar 1.4) by Armstrong, Rowe, Hotchin, Lehmann-Grube and others (1945–1965).

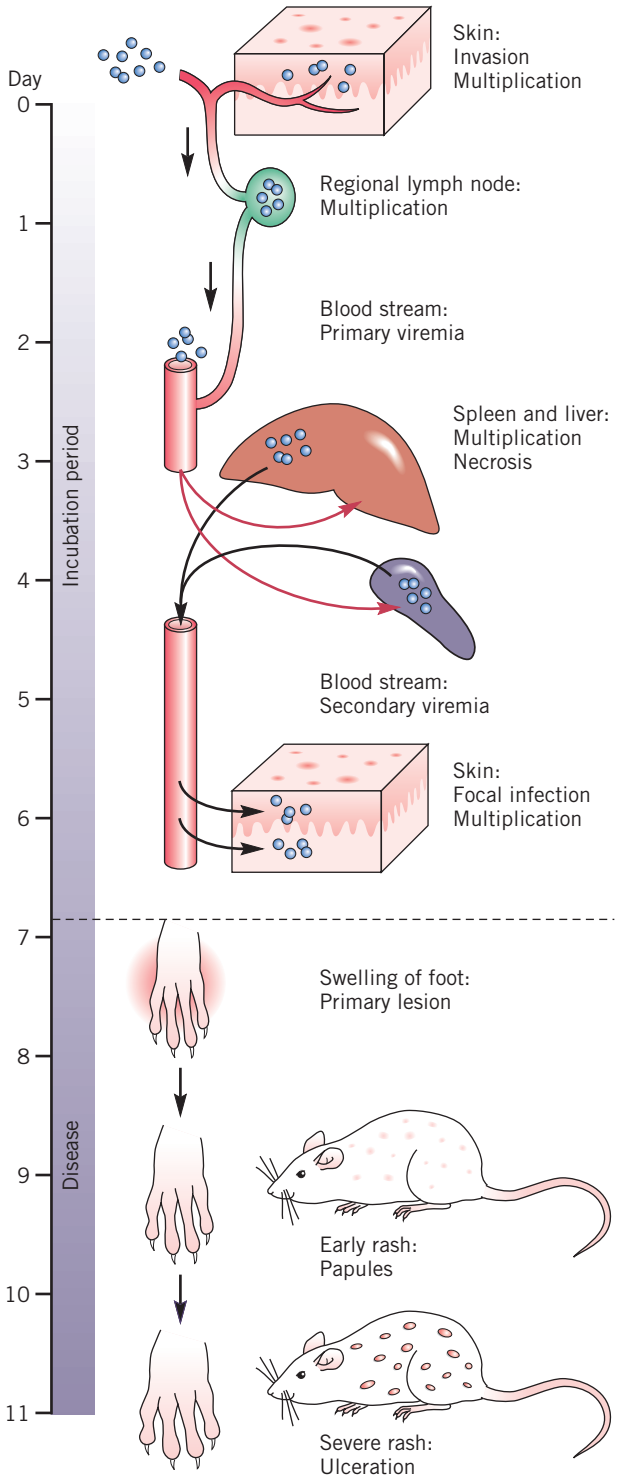

FIGURE 1.2 The spread of ectromelia virus after intradermal infection of a mouse. Redrawn from Fenner F. The pathogenesis of the acute exanthems: an interpretation based on experimental investigations with mousepox (infectious ectromelia of mice). *Lancet* 1948, 2: 915–920, with permission.

SIDEBAR 1.4

Lymphocytic choriomeningitis virus (LCMV) infection of mice: an immunologist's treasure chest

Lymphocytic choriomeningitis virus was originally discovered in the 1930s as a virus isolated from a few human cases of aseptic meningitis that could be transmitted to mice. It has not turned out to be of importance as a human pathogen, but has been used as a model to elucidate many basic principles of cellular immunity, discoveries that were of sufficient importance to warrant two Nobel prizes (McFarlane Burnet, 1960; Zinkernagel and Doherty, 1996). Amazingly enough, this obscure mouse virus has continued, over a period of almost 50 years, to yield important information about cellular immunity, viral persistence and the interplay between virus and host. Some of the lessons learned:

- If exposed in utero or infancy, mammals may become 'tolerant' of foreign antigens and fail to mount an immune response to them.
- T cell responses to antigens are 'restricted' by the host's MHC (major histocompatibility complex), which was subsequently explained by the fact that the T cell receptor recognizes antigenic epitopes (small peptides) bound to heterodimeric molecules encoded by the MHC.
- Antiviral immune responses may cause disease, due to the cytolytic attack of virus-specific CD8+ T lymphocytes that recognize host cells presenting MHC molecules bearing viral epitopes.
- Antiviral antibodies may also cause disease in persistently infected animals, by the accumulation of antigen–antibody complexes, that accumulate in the kidney causing chronic and eventually fatal glomerulonephritis.
- Slight molecular differences in strains of LCMV may modulate attachment to cellular receptors, with profound impact upon the course of infection, depending on whether or not the virus infects and kills professional antigen-presenting cells.
- Transient extraneous immunosuppression, such as produced by drugs, can convert an acute, potentially lethal LCMV infection into a persistent tolerant infection, providing a model for acceptance of transplanted tissues and organs.

THE ERA OF MOLECULAR BIOLOGY: 1975–2000

With the advent of molecular biologic methods, it became possible to sequence viral genomes and to modify them in order to determine the genetic basis of viral variation and virulence. Applied to in vivo studies, viral genomes and their transcripts could be visualized, using in situ hybridization and the in situ polymerase chain reaction (PCR). Starting in the 1990s, it became possible to manipulate the genomes of mammalian hosts, either by ablating a specific gene function ('knockout') or by inserting new or altered genes (transgenic animals) and these techniques have been used to tease apart the components of the host response, both those that protect and those that lead to disease.

Methods in immunology, particularly cellular immunology, have been radically upgraded in the 1990s, reflecting several important developments: first, an increased sophistication of flow cytometry that permits the separation and analysis of subpopulations of lymphocytes based on an array of surface markers; second, the discovery of an assortment of cytokines that transmit information among lymphocytes and monocytes; and third, the rapidly evolving field of molecular cell biology which has revealed a wide array of complex intracellular signalling pathways.

Currently, techniques in cell biology, immunology, molecular biology and genetics, as well as virology, are being exploited for the understanding of specific problems in viral pathogenesis. A few selected examples will illustrate the advances made with these newer methods.

Live attenuated strains of poliovirus (oral poliovirus vaccine, OPV) were licensed for use as vaccines in the USA in 1961 and were widely used in the years that immediately followed. Epidemiological surveillance soon documented that vaccine-associated cases of poliomyelitis were occurring in both vaccine recipients and in their immediate contacts. OPV is administered by feeding, replicates in the intestine and is excreted in the stool. When virus isolates from recently vaccinated subjects were tested in cell culture or in monkeys for markers of attenuation, it was apparent that many isolates exhibited a phenotypic reversion to virulence.

The genetic sequences of the attenuated poliovirus vaccine strains differ at a number of sites from their virulent parent strains. Testing of chimeric viruses, constructed by substituting patches of avirulent genomes into the genetic 'backbone' of virulent viruses, identified about 10 critical bases that were vital for attenuation, spread across the 7000 base genome. As shown by Minor, Almond and Racaniello, the reversion from attenuated to a more virulent phenotype after OPV feeding to humans was due to the selection of virus clones with mutations at several of these critical sites (discussed in Chapter 9). From this information, it is now possible to explain the genetic basis for poliovirus attenuation and to construct variant strains that are less capable of reverting to a virulent phenotype after feeding to humans. Unfortunately, the complexities of licensing have made it impractical to utilize these 'safer' variant viruses.

Investigations of Rous sarcoma and other retroviruses of chickens and mice have elucidated the basis for the transforming viral phenotype which, in turn, has opened the new field of cellular oncogenes (see Chapter 11). Initial genetic studies showed that transforming retroviruses carry an open reading frame for an oncogene that confers the transforming activity upon the virus. Furthermore, transforming viruses lack genetic sequences encoding the viral envelope. As a result, these viruses are replication defective and can only be propagated in the presence of a closely related replication-competent 'helper' retrovirus that supplies the envelope protein in trans. These findings clarified the role of the helper virus, a very enigmatic

feature of transforming retroviruses that had perplexed a generation of investigators.

Bishop and Varmus, who pioneered the identification of the *src* oncogene (named after Rous sarcoma virus) in the 1970s, were surprised to find that it was similar to a host gene that encoded a cellular tyrosine kinase (Sidebar 1.5). This discovery led to the insight that viral oncogenes were derived from host genomic sequences by recombination. In the process of this genetic exchange, the majority of oncogenic retroviruses have lost part of the viral genome that encodes the viral envelope protein, explaining their need for a helper virus.

Further investigation showed that the expression and activity of the normal cellular src enzyme was controlled by a complex network of other cellular proteins involved in the cell cycle, while the viral variant escaped regulation and perturbed the cell cycle so that transformed cells were no longer subject to contact inhibition and other growth restraints. These findings initiated the discovery of oncogenes, which has revolutionized our understanding of the cell cycle and the multiple mechanisms by which cells can be released from normal control mechanisms to assume the transformed phenotype.

SIDEBAR 1.5

The discovery of a cellular homolog of the *src* oncogene

'Infection of fibroblasts by avian sarcoma virus (ASV) leads to neoplastic transformation of the host cell. Genetic analyses have implicated specific viral genes in the transforming process, and recent results suggest that a single viral gene is responsible. . . . . We demonstrate here that the DNA of normal chicken cells contains nucleotide sequences closely related to at least a portion of the transforming gene(s) of ASV; . . . Our data are relevant to current hypotheses of the origin of the genomes of RNA tumour viruses and the potential role of these genomes in oncogenesis.

'Our procedures to isolate $cDNA_{sarc}$ exploited the existence of deletion mutants of ASV which lack 10–20% of the viral genome (transformation defective or td viruses); results of genetic analysis indicate that the deleted nucleotide sequences include part or all of the genes responsible for oncogenesis and cellular transformation. . . The preparation of $cDNA_{sarc}$ used was a virtually uniform transcript from about 16% of the Pr-C ASV genome, a region equivalent in size to the entire deletion in the strain of td virus used in our experiments. . . .

'DNA from several avian species . . . contain nucleotide sequences which can anneal with $cDNA_{sarc}$ . . . In contrast, we detected no homology between $cDNA_{sarc}$ and DNA from mammals. . . We suggest that part or all of the transforming gene(s) of ASV was derived from the chicken genome or a species closely related to chicken, either by a process akin to transduction or by other events, including recombination. . . The sequences homologous to $cDNA_{sarc}$ in the genome of ASV are slightly diverged from the analogous sequences in chicken genome;. . . We anticipate that cellular DNA homologous to $cDNA_{sarc}$ serves some function which accounts for its conservation during avian speciation. The nucleotide sequences which anneal with $cDNA_{sarc}$ . . . could represent either structural or regulatory genes. . . We are testing the possibilities that they are involved in the normal regulation of cell growth and development or in the transformation of cell behavior by physical, chemical or viral agents.'

Quoted extracts from Stehelin D, Varmus HE, Bishop JM, Vogt PK. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* 1976, 260: 170–173. This report was the first evidence that viral oncogenes were derived from cellular homologs and led to the discovery of a plethora of viral and cellular genes that could transform cells and played a causal role in many types of cancer.

PATHOGENESIS IN THE NEW MILLENNIUM

The recent sequencing of the human genome and those of a number of other mammalian species has begun a new era in biology. Fueled by techniques for mapping and manipulating animal genomes, the fields of virology and immunology are focused increasingly on experiments done in animals. This represents a radical change from the reductionist and chemical approach that once was advocated by leaders in biology. In this new era of 'molecular medicine', viral pathogenesis is taking on greater prominence, reflected in the addition of sections on 'viral–cell interaction' and 'pathogenesis' in leading journals of virology.

In the few years since the first edition of this book, a large variety of technical advances in life sciences have changed the landscape for investigation of pathogenesis. A few examples will illustrate these developments.

New methods for imaging, combined with molecular approaches, have made it possible to image virus replication in a living animal, as shown in Figure 1.3.

Recent developments have made it increasingly easier to manipulate selected individual host genes, either to block their expression or to introduce transgenes whose expression is tissue-specific. RNA interference (RNAi) can be used to interfere with gene expression in vivo and lentiviral vectors can introduce transgenes that are driven by tissue-specific promoters.

Probably the most important technical development is the various methods that, in the aggregate, are often called genomics. The increasing availability of annotated host and viral genomes, the use of microarrays and proteomics to identify genes that are up- or down-regulated and the application of bioinformatics to identify patterns of gene expression, offer a new and powerful approach to problems in viral pathogenesis. An example is shown in Figure 1.4 in which the response of monkeys to experimental infection with variola (smallpox) virus is explored by using microarray to identify the expression of a large number of host genes that participate in the innate immune response to this acute infection. Relman and others have pioneered this approach to dissecting the complex host response to infectious agents.

A different use of the new technology is an initiative to identify host cell genes essential for the replication of individual viruses, described in Sidebar 1.6. In this situation, a lentiviral vector is used to ablate individual host genes in a cell culture and individual cell clones that exhibit impaired ability to support the replication of a test virus are then characterized to identify the altered gene. This approach can be used to assemble a panel of

host cell genes required for the replication of the virus of interest, a novel approach that could only be undertaken in the era of genomics.

Another initiative made possible by genomics is the effort to breed a large number (perhaps 1000) strains of mice derived by crosses from existing inbred lines. This plan (summarized in Figure 1.5) would provide a very large number of inbred mouse lines in which parental genes were 'shuffled' at random, for the analysis of complex traits, i.e. phenotypes that were determined by a number of different genes. Such animals would provide the substrate for a new era in analysis of host genes that influenced susceptibility and resistance to viral infections.

A different development is the recognition that viruses play a role in the pathogenesis of an increasing variety of chronic illnesses. In some instances, the causal

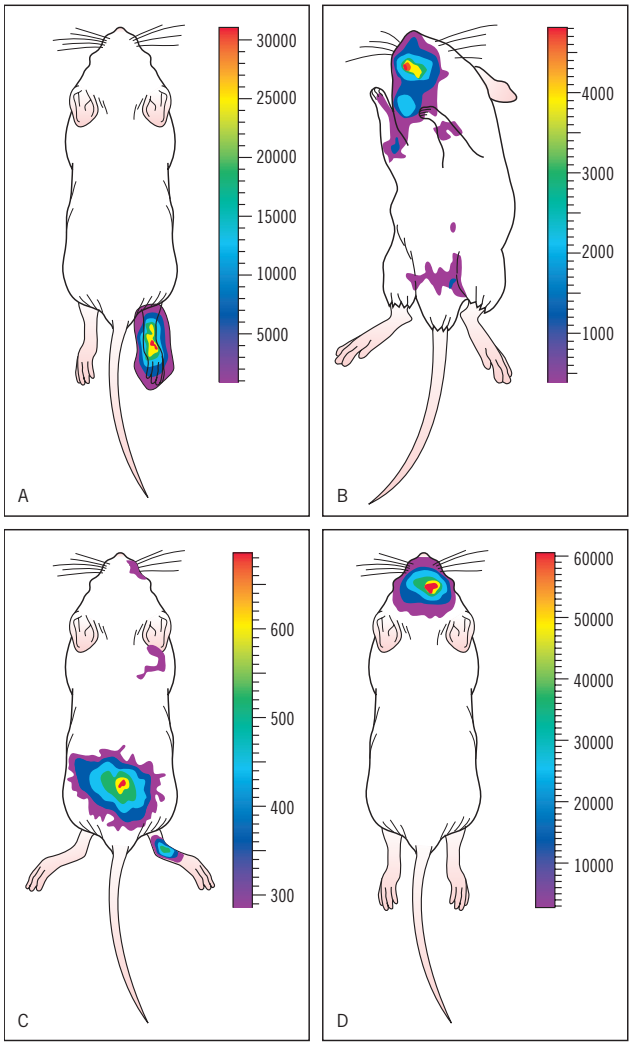

FIGURE 1.3 Tracking the spread of a neurotropic virus from the periphery to the central nervous system in an intact animal. Mice were infected by subcutaneous injection in the right footpad with a Sindbis virus that had been engineered to express, in addition to viral genes, luciferase under an internal promoter. Images A–D respectively, were taken at 8 hours and 1, 3 and 4 days after infection. They show that the virus used two pathways to the central nervous system, either up peripheral nerves to the spinal cord or via blood to the olfactory bulb and thence to the brain. After Cook SH, Griffin DE. *Journal of Virology* 2003, 77: 5333–5338, with permission.

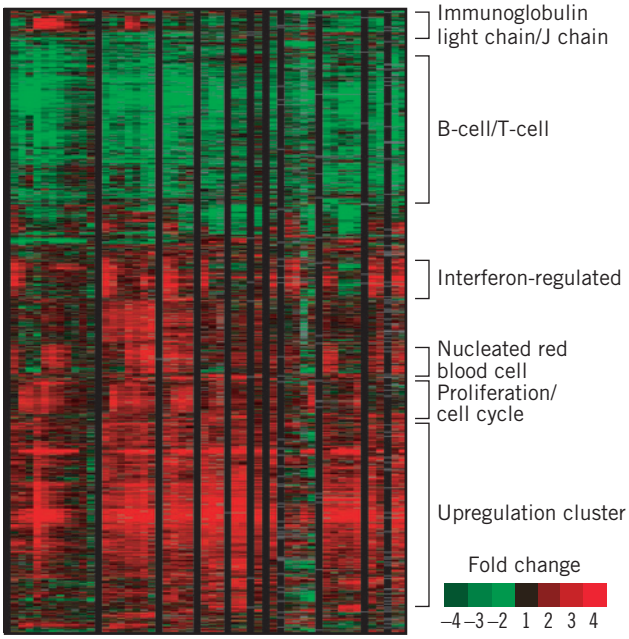

FIGURE 1.4 The response of peripheral blood mononuclear cells in monkeys infected with variola virus. Even at this low resolution, it is clear that certain groups of genes are upregulated while others are downregulated. A total of 2387 elements displayed ⩾3-fold change in mRNA expression. The data for these 2387 clones were hierarchically clustered. Data from individual elements or genes are represented as a single row and different time points are shown as columns. Red and green denote expression levels greater or less, respectively, than baseline values. Successive samples in the time course are displayed as consecutive columns. Animals are arranged from left to right based on their survival time. The seven left-hand columns represent one set of animals, and the right-hand four columns a second set of animals. After Rubins KH, Hensley LE, Jahrling PB *et al.* The host response to smallpox: analysis of the gene expression program in peripheral blood cells in a nonhuman primate model. *Proceedings of the National Academy of Sciences* 2004, 101: 15190–15195.

SIDEBAR 1.6

An initiative to identify host cell genes essential for the replication of individual viruses

1. An MMLV-derived (Moloney mouse leukemia virus) vector is used to infect a mouse cell culture (permissive for the virus under test) at a low multiplicity to favor one gene disruption per infected cell.
2. Cellular clones are derived from the MMLV-infected culture.
3. Each cell clone is tested for its ability to support replication of a selected virus.
4. Cell clones that resist viral infection are studied to identify the interrupted gene, using polymerase chain reaction (PCR) and primers based on the MMLV, to amplify and clone fragments of the interrupted gene.
5. The interrupted gene is identified by bioinformatics programs that match the cloned fragments against human and murine genomic databases.

Adapted from Murray JL, Mavraki M, McDonald NJ *et al.* Rab9 GTPase is required for replication of human immunodeficiency virus type 1, filoviruses and measles virus. *Journal of Virology* 2005, 79: 11742–11751, with permission.

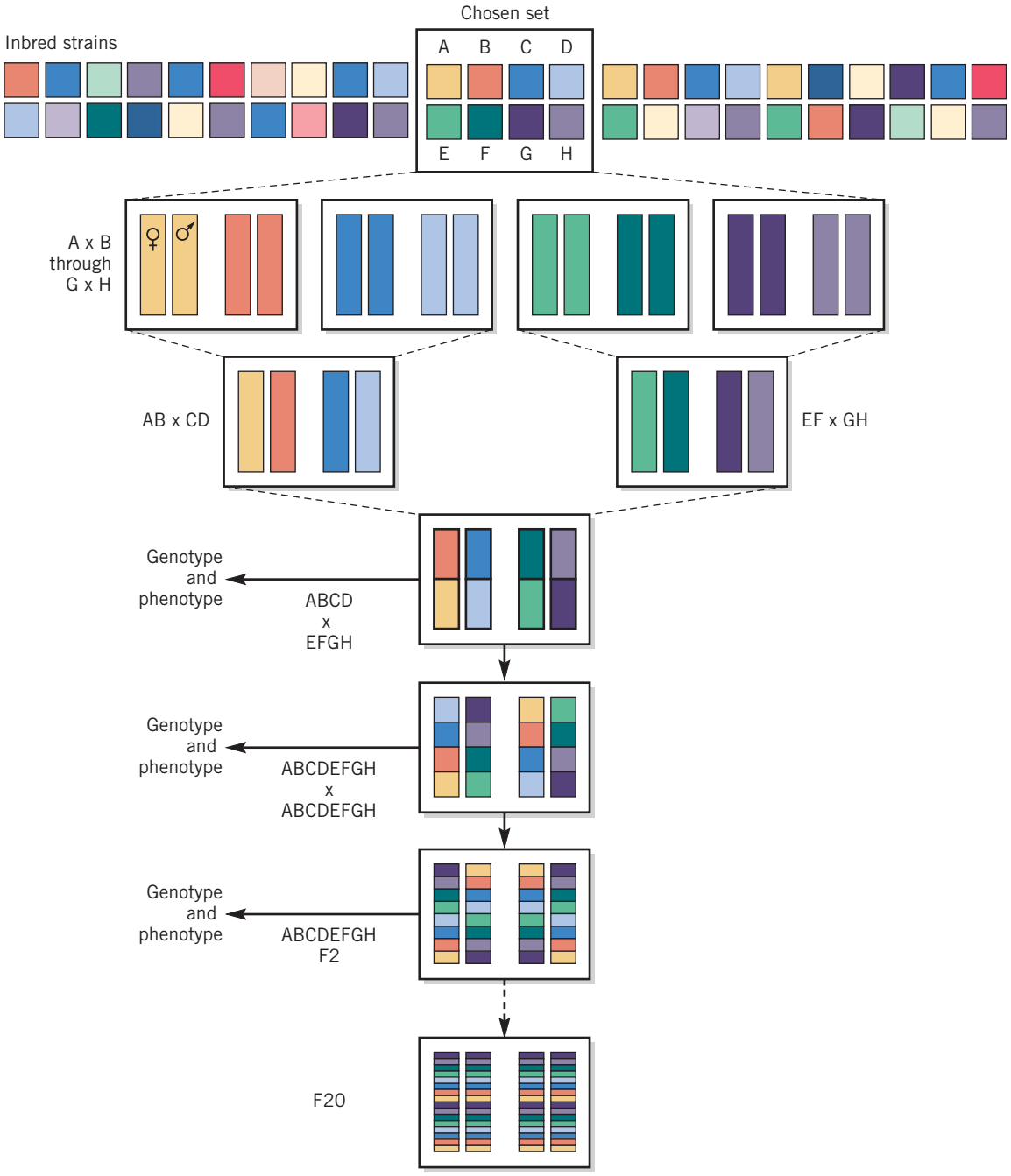

FIGURE 1.5 Proposal to develop a large number of inbred mouse strains with mixtures of genes from eight inbred progenitor lines. Mice would be outcrossed for four generations and then brother-sister pairs would be inbred for 20 generations to create new inbred strains. Adapted from Vogel G. Scientists dream of 1001 complex mice. *Science* 2003, 301: 456–457.

relationship is well established, such as the pathogenic role of human papillomaviruses in cervical cancer and the ability of certain viruses – such as JC papovavirus, the cause of progressive multifocal leukoencephalopathy – to cause chronic fatal neurological syndromes. In other instances, the evidence is insufficient to determine whether or not there is a link, for instance, multiple sclerosis and type 1 diabetes. However, it may be predicted that the list of viruses associated with chronic diseases will continue to expand, as illustrated by the recent identification of the herpesvirus that causes Kaposi's sarcoma.

It is also worthy of mention that, despite these advances in biomedical knowledge, there remain many challenging and significant unsolved problems in viral pathogenesis. For instance, it is sobering to reflect that, in the year 2007, when we have eradicated type 2 poliovirus and may be on the brink of eradication of types 1 and 3, there are still many fundamental aspects of poliovirus pathogenesis that are poorly understood. These include the initial site of enteric replication, the cellular sites of replication in lymphoid tissue, the mechanism of central nervous system invasion, the localization of virus in anterior horn cell

neurons, the role of the virus receptor in tissue tropism and the precise mechanism of cell killing. Human immunodeficiency virus (HIV) provides another example of pathogenesis and immunity that is incompletely understood, offering dozens of yet-to-be-solved questions (discussed in Chapter 14). Our knowledge of HIV is still insufficient to deal with problems of immense significance, such as the possible 'cure' of a persistent infection or the induction of protective immunity.

As we move into a new millennium, advances in biology provide a plethora of new opportunities for research in disease mechanisms, treatment and prevention. At the same time, we are confronted with an array of fundamental and applied questions which offer numerous challenges. A detailed understanding of the pathogenesis of a specific disease is essential background for the rational design of therapeutics and vaccines. This is well illustrated by the success and limitations of antiretroviral therapy for AIDS and the impediments to the formulation of an effective prophylactic vaccine for HIV. The juxtaposition of opportunities and challenges has provided a major impetus for summarizing our current knowledge of viral pathogenesis in the hope that it will provide a foundation for future research and discoveries.

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Chapter 2

CHAPTER CONTENTS

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Blood, urine, milk
Environmental survival of shed virus

TRANSMISSION

PERPETUATION OF VIRUSES
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Viruses that cause persistent infections
Control and eradication of human viruses

REPRISE

FURTHER READING

The Sequential Steps in Viral Infection

Neal Nathanson and Frederick A. Murphy

OVERVIEW

Infection of an animal host that has many specialized organs and tissues is a complex multistep process. Particular viruses usually invade at very specific sites that partly determine their subsequent route of spread, both locally and systemically, and their principal target organs and tissues. Individual viruses then spread mainly by one of two routes, either through the blood or via the peripheral nervous system. At each step in this process, the virus must overcome natural barriers to dissemination, such as the anatomic boundaries that separate organs and tissues. In addition, the restriction of replication of specific viruses to certain tissues and cells, a phenomenon often called 'tropism', can influence the apparent pattern of spread. Virus shedding can be either from the initial portal of entry or from distant sites that border on the external environment. Also, certain viruses are transmitted from the blood by transfusion, contaminated needles or blood-sucking arthropods. The following account follows the teachings of Cedric Mims, one of the pioneers of viral pathogenesis.

During acute infection, viral replication is repeatedly checked by host defenses, both non-specific and specific, such as the immune response. In many acute viral infections, the host response succeeds in eliminating the invading virus completely within a few days to weeks. However, in a number of instances, the virus manages to circumvent host defenses sufficiently to persist for varying periods of time. Although viruses vary widely in their patterns of dissemination, individual viruses tend to follow very stereotyped patterns based on properties encoded in the viral genome.

Some viruses are confined to the site of initial infection and spread only locally, while others disseminate widely. Blood-borne viruses may invade almost any organ or cell type, while neurotropic viruses are usually confined to the peripheral and central nervous system and replicate in relatively few peripheral tissues. The alternative patterns of entry, dissemination and shedding that are used by a blood-borne and a neurotropic virus are shown in Figures 2.1 and 2.2.

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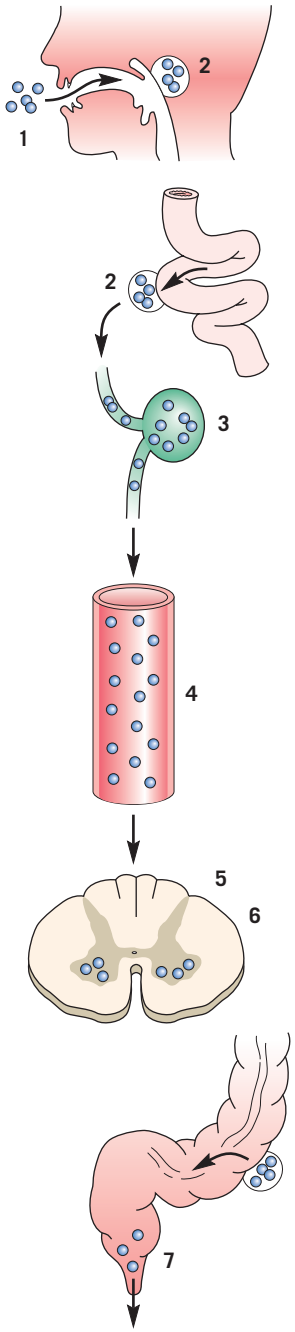


1. Virus ingested — Day 0

2. GUT ASSOCIATED LYMPHOID TISSUE — Day 0–3
- tonsils, Peyer's patches
- virus invades (via M cells?)
- replicates in monocytes

3. REGIONAL LYMPH NODES — Day 3–5
- replicates in monocytes

4. BLOOD — Day 5–15
- plasma viremia

5. BLOOD BRAIN BARRIER — Day 8–12
- virus crosses endothelium

6. SPINAL CORD
- virus replicates in anterior horn cells — Day 10–30
- cell destruction
- paralysis — Day 12–30

7. GUT — Day 5–45
- virus excreted in feces

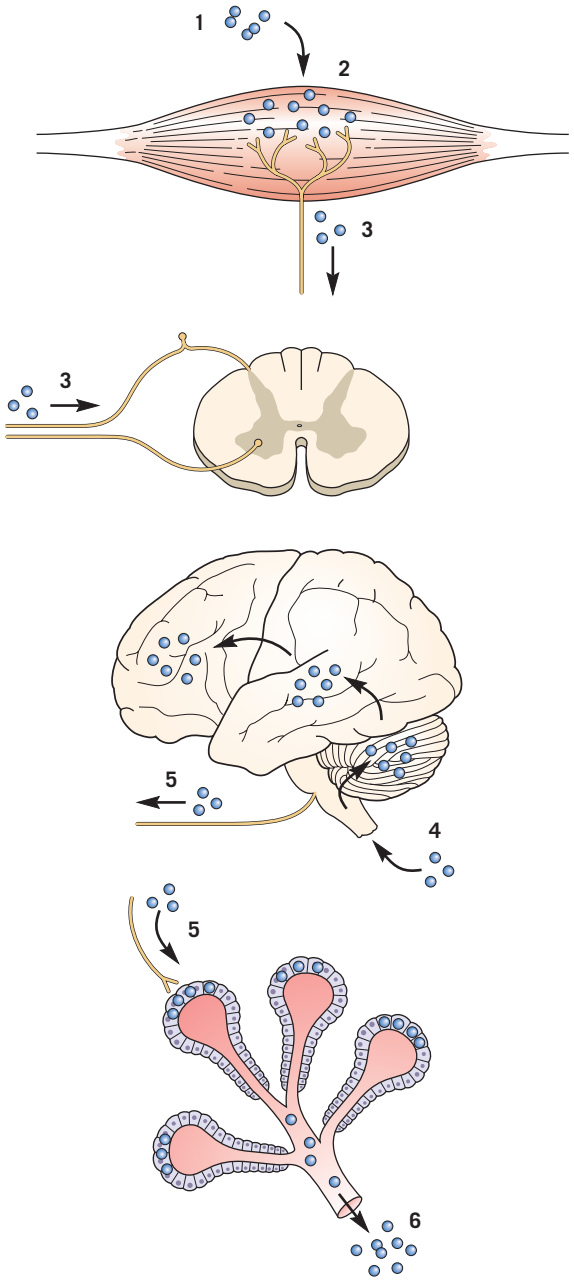
FIGURE 2.1 The spread of representative viruses. Poliovirus, an example of a virus that disseminates via the blood.

ENTRY

Skin and mucous membranes

The skin consists of the epidermis and underlying dermis. From the surface inward, the layers of the epidemis are the stratum corneum, a layer of dying cells covered by a superficial layer of keratin; the stratum granulosum; the stratum spinosum; and the stratum germinativum, a germinal layer of dividing cells that gives rise to the more superficial layers that are constantly being sloughed and replaced. Below the epidermis lies the dermis, a layer of highly vascularized connective tissue containing fibroblasts and dendritic cells (specialized macrophages).

Many different viruses replicate in cells of the skin or mucous membranes (Table 2.1). It is unlikely that any virus can invade the intact skin since there are no viable cells directly on the surface; in fact, the exterior of the skin constitutes a relatively hostile environment due to its dryness, acidity and bacterial flora. Rather, virus invades through a break in the barrier that allows contact with living cells. Skin invaders typically replicate in specific cells. For instance, both herpes simplex virus and poxviruses replicate in germinal cells of the epidermis as well as macrophages and fibroblasts of the dermis. By contrast, papillomaviruses initially infect only the germinal cells of the epidermis; however, this group of



1. VIRUS ENTRY — Day 0
- by bite of rabid animal
- infected saliva is injected

2. STRIATED MUSCLE — Day 1–60
- virus replicates in myocytes

3. PERIPHERAL NERVES — Day 10–60
- virus enters nerve ending
- nucleocapsid carried by fast axoplasmic flow to spinal cord

4. CENTRAL NERVOUS SYSTEM — Day 12–60
- virus travels along neural processes, spreads and replicates
- neuronal dysfunction
- clinical rabies, death — Day 50–70

5. PERIPHERAL NERVES — Day 30–70
- virus travels along peripheral nerves from CNS
- invades salivary gland

6. SALIVARY GLAND — Day 40–70
- virus replicates in acinar cells
- virus is discharged in saliva

FIGURE 2.2 The spread of representative viruses. Rabies virus, an example of a virus that spreads by the neural route only.

viruses cannot mature in germinal cells and complete their replication cycle in the stratum granulosum.

Most superficial invaders can infect epithelial cells on the surface of mucous membranes, although they must first penetrate a mucus barrier that may contain IgA and other virocidal proteins. The conjunctiva of the eye, a specialized mucous membrane, is the primary site of entry of a few viruses, such as certain adenovirus types and selected enteroviruses (such as coxsackievirus A24 and enterovirus 70) that can cause conjunctivitis.

Transcutaneous injection

Some viruses breach the cutaneous barrier by injection. A wide variety of viruses are arthropod-borne (arboviruses) and have a life cycle that alternates between an insect vector and a vertebrate host. These agents are injected by the infected insect when it takes a blood meal, which involves probing for a capillary with consequent injection of virus-contaminated saliva that is deposited mainly in the subcutaneous tissues but also in the circulation. It is estimated that during feeding a mosquito deposits $\sim 10^{-4}\mu l$ of saliva, which would contain 100 plaque forming units (PFU) of virus if the saliva titer is 10^9 PFU per ml.

Viruses may also be injected in other ways. Rabies virus and B virus (an α-herpesvirus of non-human primates) are often transmitted by bite of an infected animal; in this instance infection is initiated by intramuscular inoculation of virus-contaminated saliva. Several medically important viruses (hepatitis B virus

Site of entry	Route	Virus family	Representative example
Skin	Minor breaks	Papillomaviridae	Human papilloma virus
		Herpesviridae	Herpes simplex virus 1
		Poxviridae	Ectromelia virus
Conjunctiva	Contact	Picornaviridae	Enterovirus 70
		Adenoviridae	Adenoviruses
Oropharynx	Contact	Herpesviridae	Epstein-Barr virus
Genital tract	Contact	Retroviridae	HIV
		Papillomaviridae	Human papilloma virus
		Herpesviridae	Herpes simplex virus 2
Rectum	Contact	Retroviridae	HIV

TABLE 2.1 Representative viruses that invade via skin and mucous membranes
Modified after Mims CA, White DO. *Viral pathogenesis and immunology*, Blackwell, Oxford, 1984.

Localization of disease	Replicate in the pharynx and/or gastroenteric tract	Virus family	Representative example
Gastroenteritis	Yes	Astroviridae	Astroviruses
	Yes	Caliciviridae	Norwalk virus
	Yes	Coronaviridae	Transmissible gastroenteritis virus of swine
	Yes	Rotaviridae	Rotaviruses
	Yes	Parvoviridae	Canine parvoviruses
	Yes	Adenoviridae	Adenoviruses 40, 41
No enteric illness (±systemic illness)	Yes		
	Yes	Picornaviridae	Poliovirus
	Yes	Picornaviridae	Coxsackieviruses
	Yes	Picornaviridae	Enteroviruses
	No	Picornaviridae	Hepatitis A virus
	Yes	Adenoviridae	Adenoviruses

TABLE 2.2 Representative enteric viruses that do and do not cause gastroenteritis
Modified after Mims CA, White DO. *Viral pathogenesis and immunology*, Blackwell, Oxford, 1984.

(HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV)) are frequently transmitted by blood or blood products or by contaminated needles.

Urogenital tract

Viruses that are sexually transmitted fall into two entry types. Some, such as herpes simplex virus type 2 and papillomaviruses, replicate in mucous membranes of the genital tract following the pattern described above. Other sexually transmitted viruses, such as HBV and HIV, which do not replicate in epithelial cells, are associated with persistent viremia and may be transmitted via minute 'injections' of blood during sexual contact. HBV may transit mucous membranes directly to invade the circulation through surface capillaries. HIV infects CD4+ T lymphocytes, macrophages and dendritic cells in the skin and submucosal tissues and is then carried to draining lymph nodes.

Oropharynx and gastrointestinal tract

The oropharynx and gastrointestinal tract are the portal of entry for many viruses; particular viruses may invade at specific sites ranging from the tonsils to the colon (Table 2.2). Some enteric invaders remain confined to the

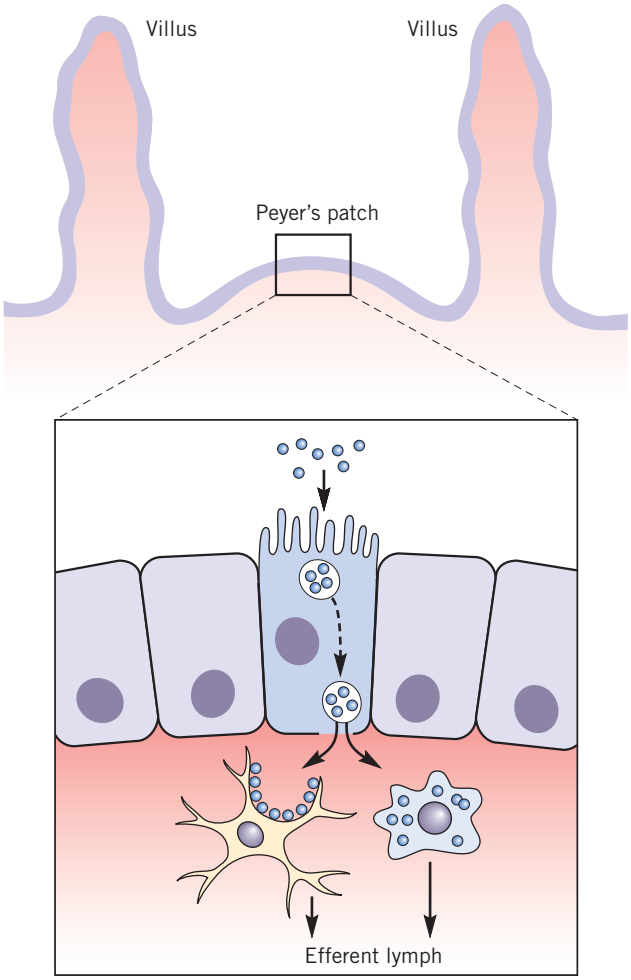


FIGURE 2.3 Virus invasion of the intestine, showing the pathway taken by reovirus in the mouse. The virus binds to M cells, is carried by transcytosis to the basolateral surface where it infects dendritic cells and macrophages in the lamina propria. This well-studied experimental model probably resembles many natural infections. After Wolf JL *et al.* Intestinal M cells: a pathway for entry of reovirus into the host. *Science* 1981, 212: 471–472.

intestinal tract, while others spread via the blood to produce systemic infection. Viruses that replicate in the gastrointestinal tract may or may not produce enteric disease.

The intimate details of entry are not well characterized for many enteric viruses, but are quite well established for reoviruses (Figure 2.3). In the alkaline environment of the small intestine, reovirions are converted to infectious subvirion particles that attach to M (microfold) cells, which form part of the specialized epithelium that overlies Peyer's patches, focal accumulations of lymphoid tissue in the wall of the intestine. Virions are endocytosed into M cells and appear to transit these cells within vesicles, to be released by exocytosis on the basal surface. From this point, virions may invade other intestinal epithelial cells through their basal surface or may be taken up by macrophages or endings of the autonomic nervous system. Different reovirus types disseminate through the circulation or along peripheral neural pathways and dissemination phenotypes have been mapped to specific viral genes.

Although most enteric viruses replicate only in the intestinal tract, some, such as poliovirus, also infect the tonsils. By contrast, other viruses, such as HIV and HBV, can invade via the rectum or colon, as indicated by the importance of anal intercourse as a risk factor for infection.

Barriers to infection

There are many barriers to infection via the gastrointestinal tract. Invading virus may remain sequestered within the intestinal contents or fail to penetrate surface mucus. The acidity of the stomach and alkalinity of the intestine, the proteolytic enzymes secreted by the pancreas, the lipolytic activity of bile, the neutralizing action of secreted IgA and scavenging macrophages, can all reduce viral infectivity. Thus, viruses that successfully utilize the gastrointestinal portal of entry must be resistant to this hostile environment or actually exploit it by activation into an infectious particle, as in the case of reovirus. There are a few viruses, such as coronaviruses, that are susceptible to this hostile environment but, when ingested in milk or food, are sufficiently protected to initiate infection by the enteric route.

Respiratory tract

Many viruses utilize the respiratory portal of entry (Table 2.3) and are acquired by aerosol inhalation or by mechanical transmission of infected nasopharyngeal secretions. Depending upon their size, aerosolized droplets are deposited at various levels in the respiratory tract. Droplets $>10\,\mu m$ in diameter lodge in the nose, those 5–10 μm in diameter lodge in the airways and those $<5\,\mu m$ reach the alveoli of the lower respiratory tract. The respiratory tract offers several barriers to invading organisms, including the protective coating action of mucus, the ciliary action of the respiratory epithelium that sweeps particles out of the airways and the activity of immunoglobulins and macrophages that engulf foreign particles. In addition, there is a temperature gradient between the nasal passages (33°C) and the alveoli (37°C) that plays an important role in the localization of infection. Thus, rhinoviruses, which infect the nasopharynx and cause the common cold, replicate well at 33°C but grow poorly at 37°C, while influenza virus, which infects the lower respiratory tract, shows the inverse temperature preference. Temperature sensitivity has been used to select attenuated influenza vaccines, since cold adapted viruses are much less virulent but replicate sufficiently in the upper respiratory tract to induce immunity against wildtype influenza virus challenge.

The initial sites of infection have been characterized for some respiratory viruses. Rhinovirus has been shown to replicate in the epithelial lining of the nose, while poxviruses, some of which enter via aerosol transmission, replicate initially in macrophages free in the airways and then in the epithelial lining of small bronchioles. By contrast, those types of reovirus that can enter via the respiratory route infect M cells that overlie bronchus-associated lymphoid tissue.

Localization of disease	Virus family	Representative example
Upper respiratory	Picornaviridae	Rhinoviruses
	Adenoviridae	Adenoviruses
Lower respiratory	Coronaviridae	Bovine coronaviruses
	Orthomyxoviridae	Influenza viruses
	Paramyxoviridae	Respiratory syncytial virus
	Bunyaviridae	Sin Nombre virus
No respiratory illness (±systemic illness)	Togaviridae	Rubella virus
	Paramyxoviridae	Mumps virus
	Bunyaviridae	Hantaan virus
	Arenaviridae	Lassa fever virus
	Reoviridae	Reovirus
	Papovaviridae	Murine polyomavirus
	Herpesviridae	Varicella zoster virus
	Poxviridae	Variola virus

TABLE 2.3 Representative viruses that invade via the nasopharynx or respiratory tract, according to localization of disease
Modified after Mims CA, White DO. *Viral pathogenesis and immunology*, Blackwell, Oxford, 1984.

SPREAD

Local spread

Viruses can be divided into two groups: those that spread only locally from their site of entry and those that disseminate widely (see Figure 2.1). Local spread occurs by infection of contiguous cells and can result in lesions such as the cold sores produced by herpes simplex virus. Epithelial cells have 'polarized' plasma membranes and certain proteins are targeted almost exclusively to either the apical or the basolateral surface. When epithelial cells are infected, virus may be released through the apical surface, in which instance it tends to remain localized, or through the basolateral surface, in which case it may disseminate more widely. Released virus is often carried from epithelial surfaces via afferent lymphatic channels to regional lymph nodes. If the virus can replicate in one of the cell types found in the node, such as monocytes or T and B lymphocytes, it is likely to disseminate via the thoracic duct into the blood.

Viremia

Viremia is the most important mode of viral dissemination within the host and can spread infection to any organ or tissue. In the blood, a particular virus circulates either free in the plasma or is cell-associated and these two kinds of viremia have different characteristics and implications. Most viremias are acute, lasting no more than 1–2 weeks, but certain viruses are able to evade immune defenses and persist in the blood for months or years.

During the course of viremia different sequential phases can be distinguished. In order to follow these events, experimental models have been used to elucidate natural infections (Figure 2.4). When a virus is injected by intramuscular, intravenous, intracerebral or other routes, a portion of the injected bolus enters the circulation without any intervening replication stage and produces a very short-lived passive viremia of a few hours duration. If the

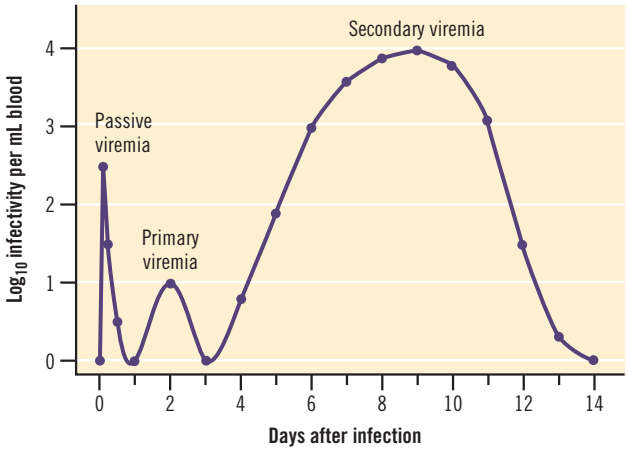


FIGURE 2.4 Stages in acute viremia, a reconstruction from experimental observations. Although this reconstruction is based on intraperitoneal or footpad injection, it likely mimics the events that follow natural routes of infection. *Passive viremia*: unreplicated inoculum entering the circulation after intraperitoneal injection of La Crosse virus. *Primary viremia*: virus entering blood after local replication following footpad injection of a small inoculum of ectromelia virus. *Secondary viremia*: virus entering blood from widely dispersed sites of replication after footpad injection of ectromelia virus. After Fenner F. The pathogenesis of the acute exanthems: an interpretation based on experimental investigations with mousepox (infectious ectromelia of mice). *Lancet* 1948, 2: 915–920 and Pekosz A *et al.* Protection from La Crosse virus encephalitis with recombinant glycoproteins: role of neutralizing anti-G1 antibodies. *Journal of Virology* 1995, 69: 3475–3481.

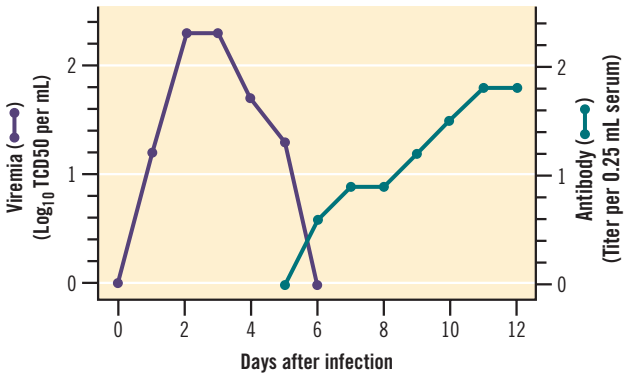


FIGURE 2.5 The course of viremia in monkeys injected by the intramuscular route with wildtype poliovirus. After Nathanson N, Bodian D. Experimental poliomyelitis following intramuscular virus injection. *Bulletin of the Johns Hopkins Hospital* 1961, 108: 320–333, with permission.

SIDEBAR 2.1

Plasma viremia: measurement of mean transit time (t_m)

In a plasma viremia, virus is constantly entering and being removed from the vascular compartment. The average duration of an infectious virion in the vascular compartment is the transit time (t_m), a parameter that differs for different viruses and is important because it determines the titer of virus in the plasma. The t_m can be measured using the formula below.

If

Vi = virus entering into the vascular compartment
Vo = virus leaving the vascular compartment
[V] = concentration of virus in the vascular compartment
t_m = mean duration of virus particles in the vascular compartment

then, at steady state, the rate of removal equals the rate of entry

$$dVi/dt = dVo/dt = [V]/t_m$$

$$\text{and } t_m = [V]/dVi/dt$$

t_m can be measured by intravenous infusion of an uninfected animal with a suspension of virus (dVi/dt) and determining the level of viremia ([V]) that is reached after several hours of infusion when a steady state is achieved. After Nathanson N, Harrington B. Experimental infection of monkeys with Langat virus. II. Turnover of circulating virus. *American Journal of Epidemiology*, 1967, 85: 494–502, with permission.

virus replicates locally at the site of entry or in the draining regional lymph node, then a brief active primary viremia may occur, lasting 1–2 days. The primary viremia serves to disseminate the virus systemically to permissive cells in various tissues; when virus is released from these secondary sites of replication an active secondary viremia occurs. This sequence is illustrated in Figure 2.4, but it should be noted that it is often hard to document these different stages in viremia except in carefully studied experimental models.

Sources of viremia

Secondary viremia can have many sources, depending on the individual virus. Those viruses that replicate in regional lymph nodes are shed into efferent lymphatics and are transported via the thoracic duct into the circulation. Some viruses replicate in the vascular endothelium and are released directly into the circulation. A number of viruses replicate in monocytes, B cells or T cells to create a cell-associated viremia; in some cases, virus may also be released from these cells to produce a concomitant plasma viremia. Viruses that replicate in other tissues, such as striated muscle or liver, may enter the vascular compartment by crossing endothelium into capillaries or via the draining lymphatics.

Plasma viremia

In acute virus infections, plasma viremia often appears within a week after infection, lasts for 1–2 weeks and comes to an abrupt termination concomitant with the appearance of circulating antibody, as shown in Figure 2.5. Viremia may be prolonged if animals are immunosuppressed, demonstrating the role of the virus-specific immune response in terminating viremia.

Plasma viremia is dynamic: virus continually enters the circulation and is continually being removed. Viral clearance is mediated primarily by the sessile macrophages of the liver, spleen and lung, which monitor the circulation for foreign particulates. The rate of clearance of virus can be expressed as the mean survival time of virus particles (t_m) within the vascular compartment (Sidebar 2.1) and, for many viruses, the t_m varies between 10 and 30 minutes. The titer of virus in the plasma is determined by the t_m and by the rate at which virus enters the circulation and can vary from trace levels to $>10^6$ infectious units per ml plasma.

Although plasma viremias are usually short lived, there are some exceptions, due to two mechanisms. In some instances, antiviral antibodies bind to circulating virus, but the immune complex retains its infectivity. Evidence for the circulation of infectious immune complexes is that the titer of plasma virus can be reduced by treatment with antisera directed against the host's immunoglobulins. Examples are lactic dehydrogenase virus infection of mice and Aleutian disease viremia of mink. Under special circumstances, the infected host may fail to recognize viral proteins as foreign (a state called 'tolerance') and fail to induce serum neutralizing antibodies. Tolerance is usually associated with infections acquired in utero or shortly after birth, prior to maturation of the immune system. Examples are HBV infection of humans and lymphocytic choriomeningitis virus (LCMV) infection of mice.

Cell-associated viremia

Some viruses replicate in cells found in the circulation, particularly B or T lymphocytes or monocytes or (rarely) erythrocytes (Table 2.4), but usually each virus infects only a single cell type. Cell-associated viremias may persist over months to years, although the titers are often low so that isolation of virus requires cultivation of blood mononuclear cells with highly susceptible indicator cells. Virus-infected cells in the blood are often shielded from attack by virus-specific cytolytic T cells or complement fixing antibodies because the viral genome is latent or is so

Cell type	Virus family	Representative example	Duration of viremia
Monocytes	Flaviviridae	Dengue viruses	Acute
	Togaviridae	Rubella virus	Acute
	Coronaviridae	Mouse hepatitis virus	Acute
	Orthomyxoviridae	Influenza viruses	Acute
	Paramyxoviridae	Measles virus	Acute
	Arenaviridae	LCMV	Persistent
	Retroviridae	HIV	Persistent
	Herpesviridae	Cytomegalovirus	Persistent
	Poxviridae	Ectromelia virus	Acute
B lymphocytes	Retroviridae	Murine leukemia virus	Persistent
	Herpesviridae	Epstein-Barr virus	Persistent
T lymphocytes	Retroviridae	HIV	Persistent
		HTLV-I	Persistent
	Herpesviridae	Human herpes viruses 6, 7	Acute
Erythroblasts	Reoviridae	Colorado tick fever virus	Acute

TABLE 2.4 Representative viruses that replicate in blood cells
LCMV: lymphocytic choriomeningitis virus; HIV: human immunodeficiency virus; HTLV: human T cell leukemia virus. Modified after Nathanson N, Tyler KL. Entry, dissemination, shedding, and transmission of viruses, in Nathanson N *et al.* (eds), *Viral pathogenesis*, Lippincott-Raven Publishers, Philadelphia, 1997.

poorly expressed that infected cells carry few, if any, viral proteins on their plasma membranes. In persistent cell-associated viremias, infectivity is usually not found in the plasma since any virus released from the infected cells is rapidly neutralized by antibodies. However, there are exceptions, such as HIV, which produces concurrent cell-associated and plasma viremias.

Spread of virus from blood into tissues

The route by which viruses cross the vascular wall into tissues has not been well characterized, although several pathways are probably operative (Figure 2.6). There are some localized regions where capillaries are fenestrated, offering the possibility for viral transit. One of these is the choroid plexus of the ventricles of the brain; certain blood-borne viruses, such as mumps, LCMV and visna viruses, probably cross the blood into the cerebrospinal fluid by this pathway, explaining why they replicate in the epithelial lining of the choroid plexus or of the ventricles. Some viruses have been visualized to transit the endothelial cell lining of capillaries by a process of endocytosis, transcytosis and exocytosis, to be released from the basal surface of endothelial cells. Finally, a number of viruses can actually replicate in endothelial cells, so that they 'grow' across the capillary wall.

A recent study has elucidated a potential mechanism whereby West Nile virus (WNV), a flavivirus, crosses the blood–brain barrier, based on a comparison of normal mice and mice with a knockout of toll-like receptor 3 ($TLR3^{-/-}$). TLR3 is a receptor for dsRNA (double-stranded RNA) and its binding activates the innate immune system (see Chapter 5). Following intraperitoneal injection in mice, WNV causes a viremia and then spreads to the brain with a fatality rate of 100%. Surprisingly, $TLR3^{-/-}$ mice were relatively resistant to WNV and 40% survived infection. $TLR3^{-/-}$ mice underwent a slightly higher viremia but, paradoxically, brain virus titers were much lower than normal mice (Figure 2.7). The following chain of events was reconstructed from further experiments: activation of TLR3 leads to upregulation of pro-inflammatory cytokines including TNFα; circulating TNFα increases the permeability of the blood–brian barrier with inflammation and virus invasion into the olfactory bulb followed by spread to the rest of the central nervous system (see also Figure 1.3).

Another quite different pathway is used by viruses that infect lymphocytes or monocytes. These cell types regularly traffic from the blood into tissues, so that the virus is carried in the form of virus-infected cells, a route that has been called the 'Trojan horse' mechanism. One example is HIV that is carried into the central nervous system by CD4+ lymphocytes or monocytes with subsequent infection of the microglia, which are the resident macrophages of the brain.

Neural spread

Neural spread is a process in which a virus is transmitted within the axoplasm of peripheral nerve fibers. The neural

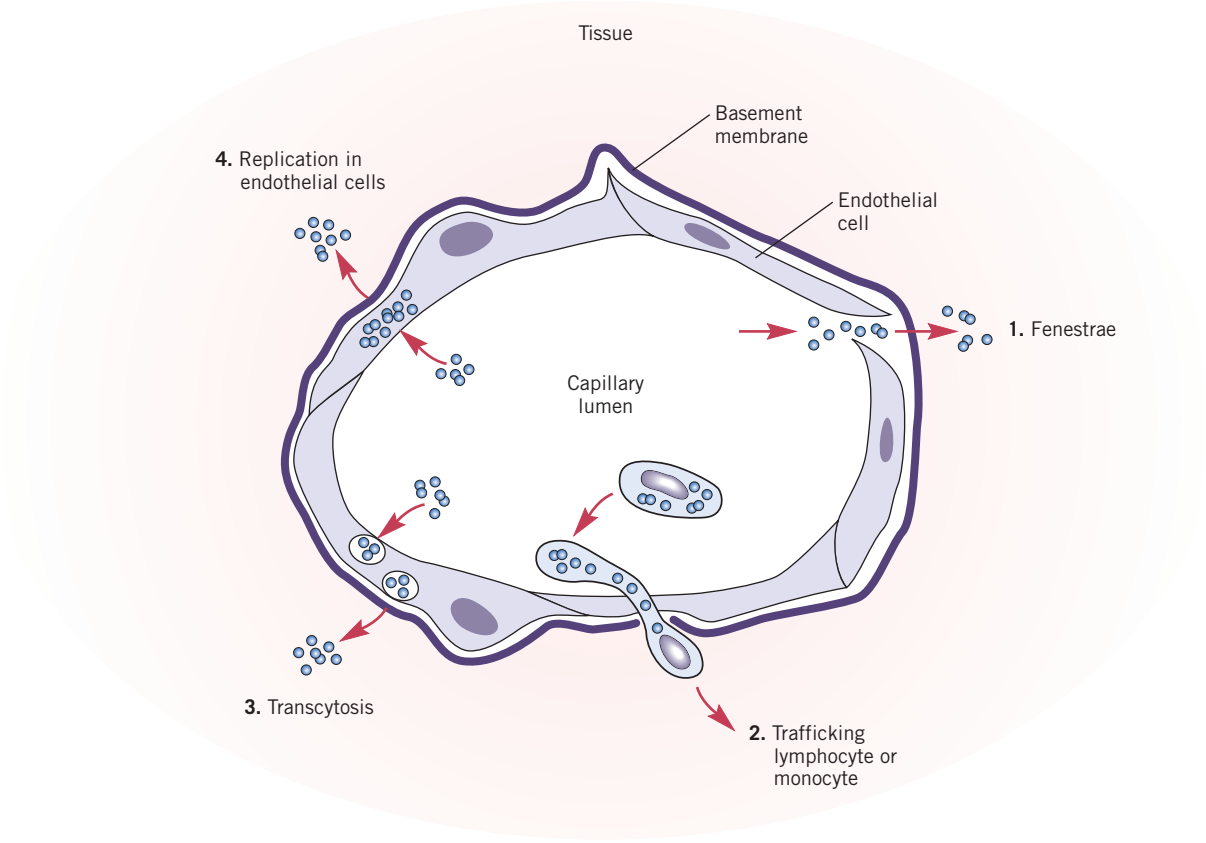


FIGURE 2.6 Viral pathways from blood into tissues.

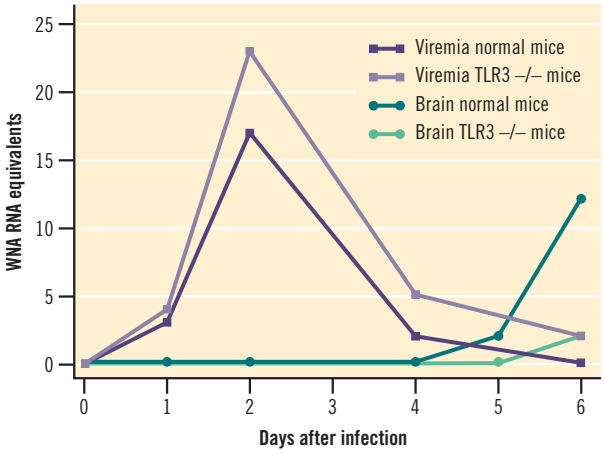


FIGURE 2.7 Pro-inflammatory cytokines can facilitate transmission of viruses from the circulation into the central nervous system. West Nile virus infection is compared in normal mice and mice with a knockout of toll-like receptor 3 ($TLR3^{-/-}$). $TLR3^{-/-}$ mice exhibit a pardoxical lower mortality (40% survival) apparently because TLR3 pro-inflammatory responses increase permeability of the blood–brain barrier. Virus titers determined by polymerase chain reaction for the envelope gene quantitated against an internal control for a tissue gene. After Wang T, Town T, Alexopoulou *et al.* Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nature Medicine* 2004, 10: 1366–1373.

pathway plays an essential role in the dissemination of some viruses, although it is less common than viremia as a mode of spread. Rabies virus is the paramount example of a virus that is an obligatory neurotrope and is not viremogenic, while α-herpesviruses (HSV (herpes simplex viruses) 1 and 2, VZV (varicella zoster virus), pseudorabies and others) are often neurotropic in adults but viremogenic in newborn humans or animals. In some instances, a virus can use both pathways, but usually this involves different viral strains with diverse biological properties. For instance, reovirus 1 is viremogenic while reovirus 3 uses the neural route. Also, it is possible to 'neuroadapt' a viremogenic virus to select a strain that uses the neural route, exemplified by the MV strain of poliovirus (Table 2.5).

The classical evidence for neural spread is the demonstration that, after viral injection into a peripheral site, a block of the innervating peripheral nerve will prevent virus from reaching the central nervous system and causing neurological disease (Table 2.5). Neural spread involves axons or dendrites and not the supporting cells, such as Schwann cells or fibroblasts that are found in peripheral nerves. Presumably, viruses enter peripheral nerve endings by the same route used to enter other permissive cells. The viral nucleocapsid is probably transported by the machinery that mediates axoplasmic flow, since viruses move at a rate (>5 cm per day) similar to that of fast axoplasmic transport (see below). Drugs, such as colchicine, that block fast axoplasmic flow, will also interfere with the neural spread of viruses. Just as axoplasmic flow is bidirectional, both toward and away from the neural cell body, viruses can spread both from the periphery to the central nervous system (CNS) and from

		Neuroadapted MV strain		Viremogenic Mahoney strain	
		Control	Nerve block	Control	Nerve block
Paralysis		25/26	0/11	19/19	18/20
Site of initial paralysis	Injected leg	24	–	3	5
	Other	1	–	16	13
Incubation to paralysis		5 days	–	7 days	7.5 days

TABLE 2.5 Different tropism of two strains of poliovirus, the neurotropic MV (mixed virus) and the viremogenic Mahoney virus. After injection into the gastronemius muscle, the MV strain spreads only by the neural route, causes initial paralysis in the injected limb and is impeded by a neural block, while the viremogenic Mahoney strain spreads by viremia, does not cause localized initial paralysis and is not impeded by nerve block. Neural block was done just prior to virus injection by freezing the innervating sciatic nerve with dry ice proximal to the site of virus injection
After Nathanson N, Bodian D. Experimental poliomyelitis following intramuscular virus injection. *Bulletin of the Johns Hopkins Hospital* 1961, 108: 308–319, with permission.

the CNS toward the periphery. Most RNA viruses can replicate within neural cytoplasm, but DNA viruses, such as herpes simplex virus, must reach the nucleus within the neuronal cell body in order to replicate.

Pseudorabies virus, a herpesvirus of pigs, has been used to study the molecular mechanism of neural spread. Herpesviruses consist of a capsid containing their DNA genome; the capsid is surrounded by a lipid envelope and there is a layer of proteins, the tegument, between envelope and capsid. The virus enters neuronal endings by fusion of the envelope with the plasma membrane of the neural cell, releasing the capsid into the cytosol. The axons and dendrites of neurons contain subcellular organelles (microtubules, kinesins, dynein) that mediate 'fast' axonal transport, a system designed to ferry subcellular components between the perinuclear region and the periphery. After entry of the herpesvirus capsid, it appears that specific viral tegument proteins tether the capsid to the proteins of the axonal transport system, that transport the capsids within the axon. The direction of transport is apparently determined by different tegument proteins that bind to anterograde or retrograde transport machinery (Figure 2.8).

Although viremia and neural spread are classically considered as alternative modes of spread, some viruses may disseminate by both routes. For instance varicella virus, an α-herpesvirus, produces a viremia, invades peripheral nerve endings in the skin and then spreads along peripheral nerves to dorsal root ganglia where it becomes latent, occasionally emerging years later in the form of herpes zoster, also called shingles. Another example is Sindbis virus, which appears to utilize both viremic and neural pathways to the central nervous system (see Figure 1.3).

Viral localization and tissue tropism are described in the next chapter.

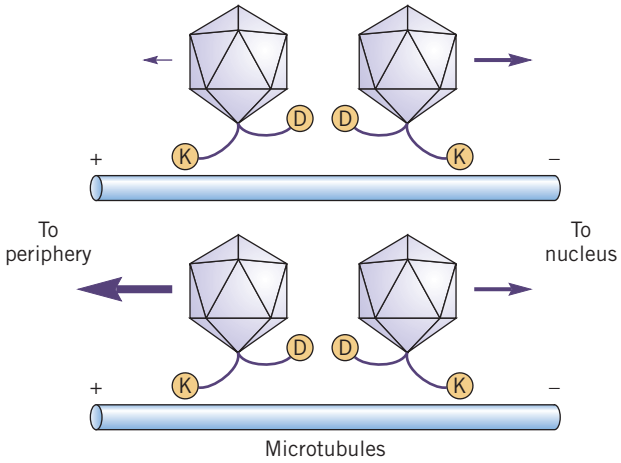


FIGURE 2.8 Hypothetical scheme to explain the movement of an α-herpesvirus within axons. Herpesvirus icosahedral capsids are shown bound to a bidirectional molecular motor system that mediates fast axonal transport. The system is composed of three main elements – microtubules that extend from the perinuclear regional through neuronal axons and two motor systems (dynein and kinesin) that move along the microtubules and can transport various molecular 'cargoes' that bind to them. Timelapse images show that viral capsids move in a saltatory fashion, i.e. they indergo a jump in one direction followed by a reverse jump in the opposite direction. Dynein (D) mediates retrograde movement (from the periphery toward the nerve cell nucleus) and is postulated to move at a constant rate. A kinesin family motor (K) moves in an anterograde direction and is postulated to move at a variable rate. In the upper diagram, retrograde movement exceeds anterograde movement and the capsid is transported from periphery towards the nucleus; in the lower diagram, anterograde movement exceeds retrograde movement and the capsid is transported from the perinuclear region to the periphery. After Smith GA, Pomeranz L, Gross SP, Enquist LW. Local modulation of plus-end transport targets herpesvirus entry and egress in sensory axons. *Proceedings of the National Academy of Sciences* 2004, 101: 16034–16039.

SHEDDING

Viruses may be discharged into respiratory aerosols, feces or other body fluids or secretions and each of these modes is important for selected agents. Viruses that cause acute infections are usually shed intensively over a short time period, often 1–4 weeks and transmission tends to be relatively efficient. Viruses, such as HBV and HIV, that cause persistent infections, can be shed at lower titers for months to years, but will eventually be transmitted during the course of a long-lasting infection.

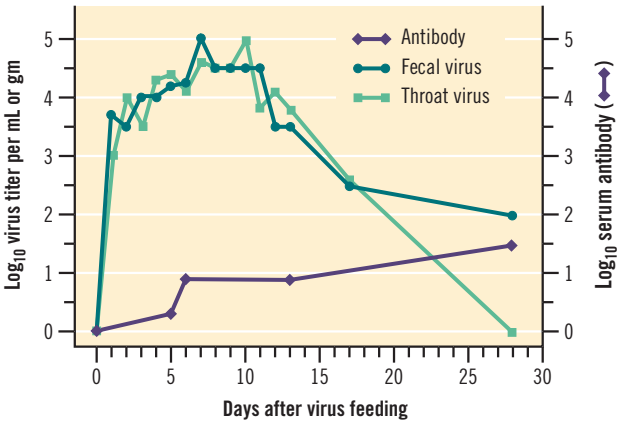


FIGURE 2.9 Course of wildtype poliovirus excretion in the pharyngeal fluids and feces of chimpanzees after virus feeding. After Bodian D, Nathanson N. Inhibitory effects of passive antibody on virulent poliovirus excretion and on immune response in chimpanzees. *Bulletin of the Johns Hopkins Hospital* 1960, 107: 143–162, with permission.

Oropharynx and gastrointestinal tract

Enteroviruses may be shed in pharyngeal fluids and feces (as shown for poliovirus in Figure 2.9). In this case, the virus replicates in the lymphoid tissue of the tonsil and in Peyer's patches (lymphoid tissue accumulations in the wall of the small intestine) whence it is discharged into the intestinal lumen. Other viruses may be excreted into feces from the epithelial cells of the intestinal tract (reoviruses and rotaviruses) or from the liver via the bile duct (hepatitis A virus).

Respiratory tract

Viruses that multiply in the nasopharynx and respiratory tract may be shed by two distinct mechanisms, either as aerosols generated by sneezing or coughing or in pharyngeal secretions that are spread from mouth to hand to hand to mouth. Often, transmission is via contaminated fomites, such as handkerchiefs, clothing or toys. Typically, viruses, such as rhinoviruses and influenza viruses that cause acute respiratory illness, are spread efficiently at high titer but for short periods, often not more than one week.

Skin

Relatively few viruses are shed from the skin, but there are some exceptions. Papillomaviruses and certain poxviruses that cause warts or superficial tumors may be transmitted by mechanical contact. A few viruses, such as variola virus, the cause of smallpox, and varicella virus, the cause of chickenpox, that are present in skin lesions, can be aerosolized and transmitted by the respiratory route. In fact, it is claimed that the earliest instance of deliberate 'biological warfare' was the introduction into the villages of hostile Indian tribes of blankets containing desquamated skin from smallpox cases.

Mucous membranes, oral and genital fluids

Viruses that replicate in mucous membranes and produce lesions of the oral cavity or genital tract are often shed in pharyngeal or genital fluids. An example is herpes simplex virus (type 1 in the oral cavity and type 2 in genital fluids). A few viruses are excreted in saliva, such as Epstein-Barr virus, a herpesvirus that causes infectious mononucleosis, sometimes called the 'kissing disease', and mumps virus. Probably the most notorious example is rabies virus, which replicates in the salivary gland and is transmitted by a bite that inoculates virus-contaminated saliva. Several important human viruses, such as HBV and HIV, may be present in the semen. It is estimated that in an HIV-infected male, an average ejaculate contains $\sim 10^6$ mononuclear cells of which 10^2–10^4 (0.01–1%) may carry the viral genome.

Blood, urine, milk

Blood is an important potential source of virus infection in humans, wherever transfusions, injected blood products and needle exposure are common (see Table 2.4). In general, the viruses transmitted in this manner are those that produce persistent viremia, such as HBV, HCV, HIV and cytomegalovirus (a herpesvirus). Occasionally, viruses that produce acute short-term high titer viremias, such as parvovirus B19, may contaminate blood products. Although a number of viruses are shed in the urine, this is usually not an important source of transmission. One exception is certain animal viruses that are transmitted to humans; several arenaviruses are transmitted via aerosols of dried urine. A few viruses are shed in milk and transmitted to newborns in that manner. The most prominent example is HIV and it appears that a few other retroviruses, such as HTLV I of humans, visna maedi virus of sheep and mouse mammary tumor virus, can be transmitted via milk.

Environmental survival of shed virus

Transmission of a virus depends both on the amount and duration of shedding and on survival in the environment, a point often overlooked. For instance, viruses differ in their ability to survive in aerosols or after drying. Thus, poliovirus is sensitive to low humidity and this is thought to account for its reduced transmission in the winter time in temperate climates where humidity is low, while transmission continues year round in tropical climates. The gastrointestinal lumen constitutes a harsh environment that can inactivate all but the hardiest viruses. Thus, of the different hepatitis viruses, all of which are probably shed in the bile, only hepatitis A virus and hepatitis E virus behave as enteroviruses, presumably because the others, such as HBV and HCV, are inactivated before they can be transmitted by the fecal-oral route.

TRANSMISSION

Following shedding, a virus can be transmitted to a new host in several different ways, but individual viruses utilize only one or two of these potential modes. The most common mode of transmission of enteric and many respiratory viruses is probably by oral or fecal contamination of hands, with passage to the hands and thence the oral cavity of the next infected host. Inhalation of aerosolized virus is also an important mode of transmission for

respiratory viruses. Another significant route is by direct host-to-host interfacing, including oral-oral, genital-genital, oral-genital or skin-skin contacts. Transmission may involve less natural modes such as blood transfusions or reused needles. In contrast to propagated infections are transmissions from a contaminated common source, such as food, water or biologicals. Common source transmission is quite frequent and can produce explosive outbreaks that range in size depending on the number of recipients of the tainted vehicle and the level of virus contamination.

Sexually transmitted viruses present a special situation, since the probability of spread depends upon the gender and type of sexual interaction between infected host and her/his uninfected contact. For instance, an HIV-infected male is more likely to transmit to a female partner via anal than vaginal intercourse and that risk is reduced if the male partner is circumcised.

Transmission of arboviruses is complex, since it involves the cycle between an insect vector (in some instances only the female takes blood meals) and a vertebrate host. There are a number of quantitative variables that determine the efficiency of vector transmission. Vertebrate host determinants include the titer and duration of viremia, while insect determinants include the competence of the vector (i.e. the ability of the vector to support viral replication in several tissues and shed virus in its saliva) and the extrinsic incubation period (the interval between ingesting the virus and shedding in the saliva), as well as the distinctive feeding preferences of each insect vector. Also, there are a number of alternative patterns of transmission, for instance the overwintering of virus in hibernating mosquitoes, the transovarial transmission of the virus and venereal spread between male and female mosquitoes. Recently, it has been shown that when an uninfected mosquito cofeeds with an infected mosquito on the same vertebrate, there may be a low rate of transmission even though the vertebrate host is not viremic.

PERPETUATION OF VIRUSES

Viruses that cause acute infections

For viruses that can only cause acute infections, transmission must be accomplished during a relatively short time frame, frequently no more than one week of shedding. The efficiency of transmission can be measured by determining the number of new infections generated by each infected host (reproduction ratio or Ro); if Ro is >1 then the agent is spreading and if it is <1 then the number of infections is declining. Although transmissibility may cycle above and below 1, overall it must be at least 1 if the agent is to be successfully perpetuated in the specified population. Acute viruses may fail to meet this criterion, in which case they 'fade out' and disappear. Measles is probably the best documented example of this phenomenon, because almost all cases of measles infection cause a readily recognized illness. Prior to measles immunization, measles periodically disappeared in populations of <500 000, only to cause an outbreak when it was re-introduced. This is dramatically illustrated in islands such as Iceland with a population of ~200 000 (Figure 2.10).

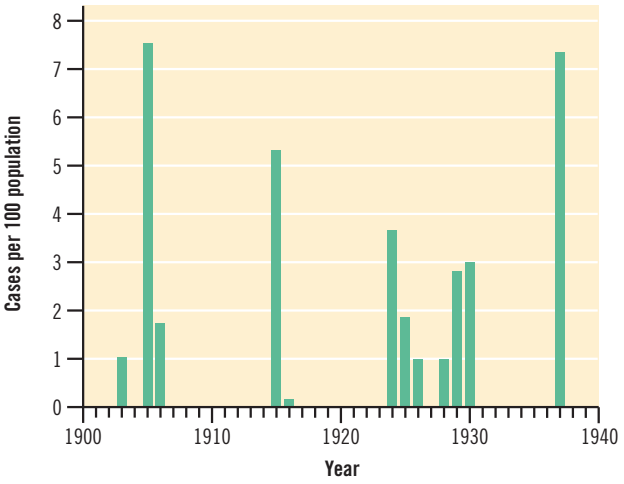


FIGURE 2.10 Measles in Iceland showing its periodic disappearance and re-introduction during the period 1900–1940, prior to the use of measles vaccine. After Tauxe unpublished, 1979 and Nathanson N, Murphy FA. Evolution of viral diseases, in Nathanson N *et al.* (eds), *Viral pathogenesis*, Lippincott-Raven Publishers, Philadelphia, 1997.

FIGURE 2.11 Antibody against hepatitis A virus in selected countries to illustrate the differences in transmissibility of a single virus in different populations. After Frosner GG *et al.* Antibody against hepatitis A in seven European countries. *American Journal of Epidemiology* 1979, 110: 63–69.

For acute viral infections, one indicator of transmissibility in a population is the age-specific prevalence of antibody, assuming that the initial infection confers lifelong detectable antibody as well as long duration immunity. Figure 2.11 shows the age-specific antibody profiles for hepatitis A in three countries, illustrating the difference in virus transmission in different populations.

Viruses that cause persistent infections

Viruses that cause persistent infections may be transmitted over a long period of time, in some cases for the lifetime of

the infected host. In this instance, perpetuation of the virus still requires that each infection must generate at least one new infection but this may take place over many years. Human viruses that behave this way include HIV, HBV and VZV and such persistent viruses can be perpetuated within very small populations. Studies of isolated primitive tribes have shown that most of the viruses that can be found are those that are capable of causing persistent infections in individual hosts, while acute viruses, when they appear, burn out very rapidly. One variant pattern of persistence is viruses that are transmitted vertically, from mother to offspring, by perinatal or transplacental routes or integrated into the host germline genes. Viruses such as lymphocytic choriomeningitis virus of mice or HTLV I or HTLV II of humans may persist in populations where there is very limited horizontal transmission.

Control and eradication of human viruses

The principles of virus shedding and transmissibility are relevant for the control and elimination of important human pathogens. Pre-exposure immunization can diminish the number of susceptible hosts in a population and reduce Ro to $\ll 1$, with the consequent disappearance of a virus from the immunized population, if virus perpetuation depends upon acute infections. This principle has been successfully applied to the global eradication of variola virus, the cause of smallpox and has led to the eradication of type 2 poliovirus. Conversely, although there is a highly efficacious vaccine for HBV, it can be calculated that it will take generations for this virus to disappear, due to persistent infections in the millions of humans who are already infected.

REPRISE

Individual viruses are very diverse in the sequential steps in infection of their mammalian hosts, from the site of invasion, degree and mode of spread, target tissues and organs and sites from which they are shed. They may invade through mucous membranes, skin, respiratory and gastrointestinal routes, as well as by injection by insect vectors or sharps. Some viruses remain relatively localized near their site of entry, while others disseminate via blood or neural routes. They may be shed into any body fluid, or from skin and mucous membranes and thence transmitted to new susceptible hosts. The mechanisms that determine these diverse patterns are the subject of subsequent chapters.

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Chapter 3

Cellular Receptors and Viral Tropism

Neal Nathanson and Kathryn V. Holmes

CHAPTER CONTENTS

WHAT IS VIRAL TROPISM AND WHY IS IT IMPORTANT?

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Viral entry

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Tropism and viral variation

REPRISE

FURTHER READING

WHAT IS VIRAL TROPISM AND WHY IS IT IMPORTANT?

Following viral infection there are many different patterns of localization and dissemination, as described in the previous chapter. The focused nature of the pathological or physiological changes caused by each virus is an attribute so characteristic that it accounts for the disease 'signature' of many viruses. Thus, smallpox was known for the rash that left survivors pockmarked for life, poliomyelitis by the paralytic attack and permanent lameness, yellow fever by acute jaundice and rhinoviruses by the common cold. Tropism is the traditional term used to refer to this anatomical localization of the signs and symptoms of a viral disease.

The mechanisms of tropism are the theme of this chapter. The most important determinants of tropism are the cellular receptors that, in general, are different for each virus group. Since receptors are unequally expressed on the cells in different tissues, they limit the possible cell types that can be infected by each virus. Following virus entry, viruses utilize a wide variety of cellular proteins for the transcription and translation of their proteins and the replication of their genomes. Again, some, but not all, cells provide the cellular proteins required for the replication of a specific virus and this further limits the range of differentiated cells in which a given virus can replicate. Finally, there are other physiological factors that constrain the replication or survival of specific viruses and can therefore influence tropism.

It is also important to recognize that there is only a partial correlation between viral replication and viral disease and that significant viral damage is often limited to a subset of the tissues in which replication occurs. For instance, poliovirus is an enteric and viremic infection but significant disease is limited to the central nervous system. Measles virus is a respiratory and viremic infection but is recognized clinically by its characteristic rash. Oncogenic viruses may replicate in several cell types but transform only one. EBV (Epstein-Barr virus) causes a productive infection of epithelial cells and a latent infection of B lymphocytes but oncogenesis is limited to B cells.

Host range or species specificity is distinct from tropism but may involve related mechanisms. Most virus groups consist of a large number of member viruses, each of which is, in nature, limited to a few host species.

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Under experimental conditions, some viruses can be readily transmitted to many host species while others are quite restricted. These issues are discussed further in Chapter 13.

VIRAL ATTACHMENT AND ENTRY

Cellular receptors for viruses

Peter Medawar described viruses as 'bad news wrapped in protein', a succinct summary of the structure of all viruses, in which the nucleic acid genome is internal to an outer protein structure that protects the genome from adverse environmental factors. The other important role of the protein coat is to deliver the viral genome across the plasma membrane to the cellular interior where replication occurs. Rapid and efficient transportation across the plasma membrane is a major engineering challenge that viruses have solved by exploiting the presence of many diverse proteins, sugars and lipids as cell surface receptors. Each virus can bind to one (or a very few) of this multitude of molecules. Viral receptors are naturally occurring cellular molecules that serve physiological functions for the cell, functions that have nothing to do with infection.

How do viruses interact with their cognate receptors? The receptor activity is due to the ability of a viral surface protein, often called the viral attachment protein (VAP), to attach to the viral receptor. Figure 3.1 illustrates the interaction in a simplified cartoon. In practice, the interaction can be more complex, as exemplified by the entry of human immunodeficiency virus (HIV) shown in Figure 3.2. Human immunodeficiency virus type 1 (HIV-1) binds to both a primary receptor (the CD4 protein) and to a coreceptor on the surface of susceptible cells. The virus will only enter and infect cells that bear both receptor and coreceptor, although there are a few special exceptions. Several different proteins, all of them chemokine receptors, can serve as coreceptors and different CD4+ cells – such as macrophages and CD4+ lymphocytes – express different coreceptors.

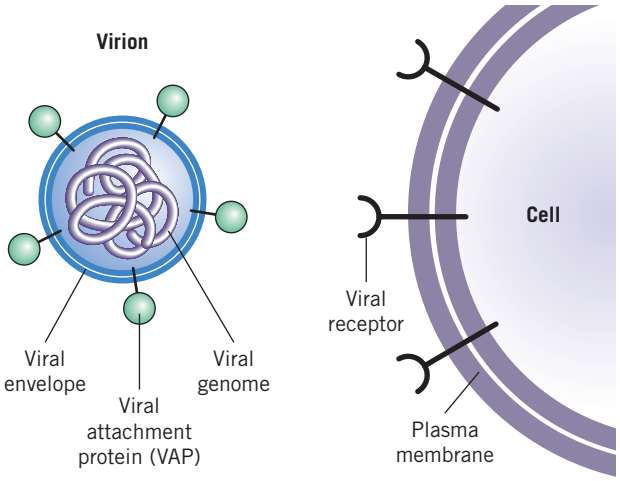

FIGURE 3.1 Diagrammatic representation of a cellular receptor and its cognate viral attachment protein (VAP). Scale has been distorted to emphasize the interaction between the VAP and its receptor.

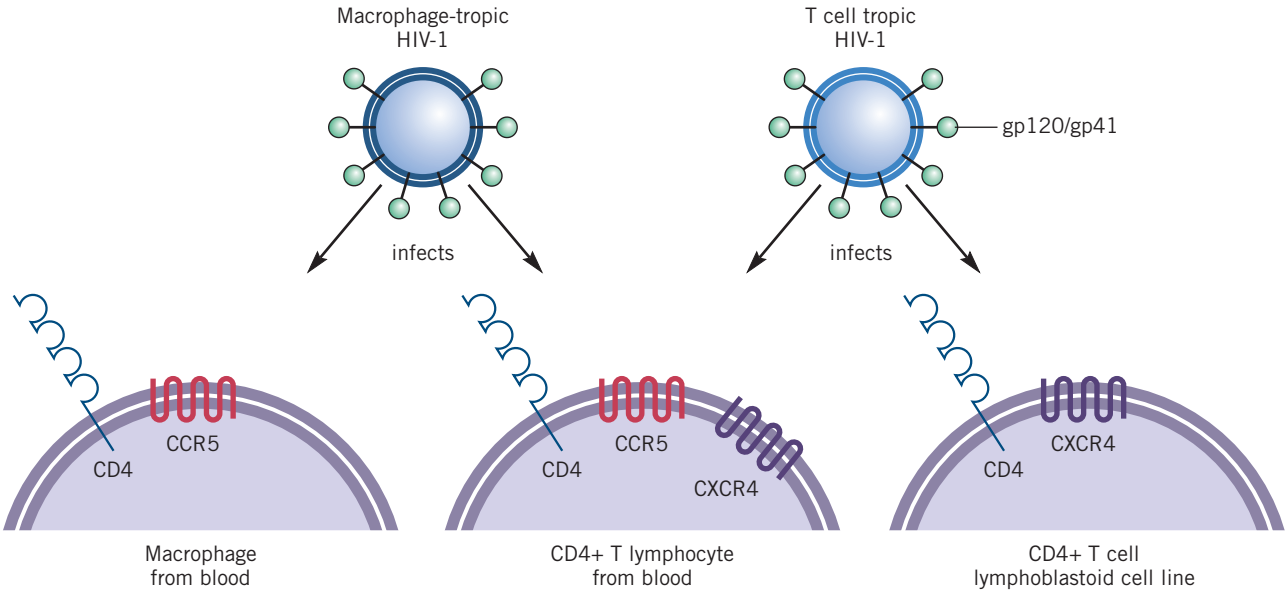

FIGURE 3.2 Cells that are permissive for the entry and replication of human immunodeficiency virus type 1 (HIV-1) carry two receptors on their surface. The primary receptor is the CD4 protein, a protein expressed on the surface of certain subsets of lymphocytes (so-called CD4+ cells). The gp120 spike glycoproteins of all HIV strains can bind to human CD4. In addition, virus entry requires a coreceptor, which is either CCR5 or CXCR4; these proteins are members of a large family of molecules that serve as chemokine receptors on the surface of lymphoid cells or macrophages. Some HIV-1 isolates are macrophage-tropic because their gp120 spikes use CCR5, a chemokine receptor that is expressed on the surface of macrophages, while other isolates are T cell-tropic because they utilize the CXCR4 molecule, another chemokine receptor expressed on the surface of T cell lines. Both kinds of viruses can replicate on peripheral blood mononuclear cells (PMBCs), a mixed population of cells that express both coreceptors. Some HIV-1 isolates (not shown) are 'dual-tropic' since they can utilize both coreceptors. (Recent studies have shown that macrophages express low levels of CXCR4 at concentrations insufficient for entry of T cell-tropic HIV-1.)

The VAP of some isolates of HIV (the envelope glycoprotein gp120) can utilize only one of the two coreceptors, producing a complex pattern of cellular susceptibility and viral host range. Another example of a virus group that uses both a primary and secondary receptor is herpes simplex viruses (HSV), the cause of 'cold sores' and similar genital lesions of humans (described below).

What cellular molecules can serve as viral receptors? Many viral receptors are glycoproteins, since most proteins expressed on the cell surface have been glycosylated during their post-translational maturation. In numerous instances, the physiological role of the viral receptor is known, but there are some cases where the normal function of the receptor is yet to be identified. Many cell surface proteins bind other soluble or cell surface proteins, thereby mediating signaling and/or cell–cell interactions. It may be speculated that viruses have 'pirated' or mimicked attachment domains of such cellular proteins to use as viral attachment proteins. Figure 3.3 shows a representative group of membrane glycoproteins that serve as viral receptors.

Glycoprotein receptors

The VAP binds to a domain that represents a small part of the surface of the glycoprotein receptor and this domain may be either a polypeptide sequence or a carbohydrate sidechain. CD4 is an example of a glycoprotein receptor whose binding domain is the amino acid backbone of the protein. CD4 is a member of the immunoglobulin superfamily of molecules that has four globular domains linked together. Mutation of CD4 has shown that the viral attachment protein of HIV (gp120) binds to a small region within the outermost globular domain of CD4. Figure 3.4 shows the structure of the outermost domain of CD4, indicating the amino acid residues that bind the virus attachment protein, gp120. Figure 3.5 shows a structural view of the interface between a VAP and its cognate receptor.

An example of a carbohydrate sidechain that acts as a receptor domain is provided by the influenza type A viruses. This receptor is sialic acid (or N acetyl neuraminic acid), a modified sugar that is found in the tips

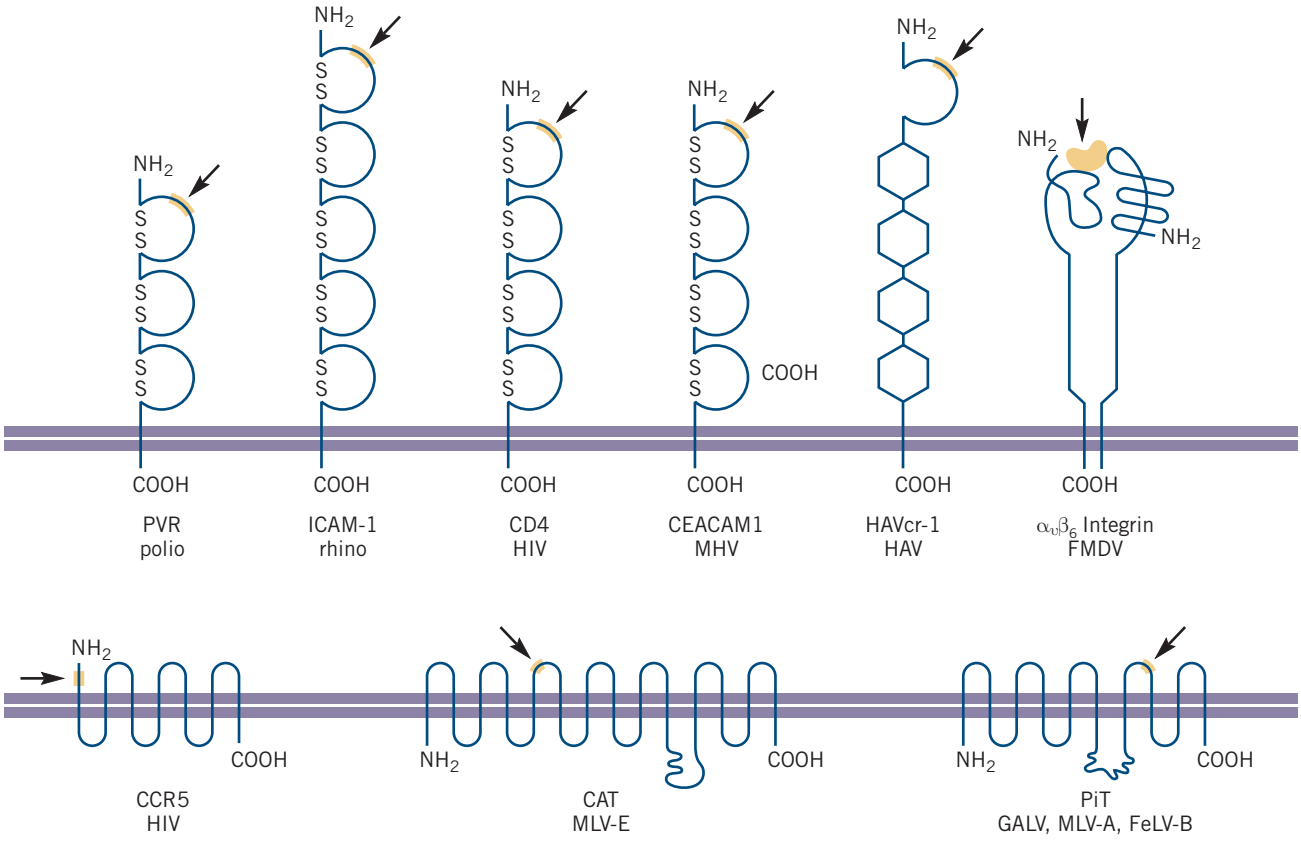

FIGURE 3.3 Molecular backbone cartoons of some glycoprotein viral receptors. Receptors vary widely in their structure and in their physiological function. The amino and carboxy termini are shown, together with important disulfide bonds and the probable domains that bind virus. Abbreviations: $\alpha_v\beta_6$: integrin chains (integrin dimers serve as receptors for many different viruses); ICAM: intercellular adhesion molecule; CCR5: chemokine receptor 5; CAT: cationic amino acid transporter; CEACAM: carcinoembryonic antigen-related cell adhesion molecule; HAVcr-1: HAV receptor cellular receptor; PiT: inorganic phosphate transporter; PVR: poliovirus receptor. Viruses: polio: poliovirus; rhino: rhinovirus, major group; FMD: foot-and-mouth disease virus; HAV: hepatitis A virus; HIV: human immunodeficiency virus; MHV: mouse hepatitis virus (a coronavirus); BLV: bovine leukemia virus; ALV-A: avian leukosis virus; MLV-E: murine leukemia virus E; GALV: gibbon ape leukemia virus; MLV-A: murine leukemia virus A; FeLV-B: feline leukemia virus B. After Holmes KV. Localization of virus infections, in Nathanson N *et al.* (eds), *Viral pathogenesis*, Lippincott Raven Publishers, Philadelphia, 1997; Wimmer E (ed.). *Cellular receptors for animal viruses*, Cold Spring Harbor Press, Cold Spring Harbor, 1994; Flint SJ, Enquist LW, Racaniello VR, Skalka AM. Attachment and entry, in *Principles of virology*, 2nd edn, ASM Press, Washington, DC, 2002; Weiss RA, Tailor CS. Retrovirus receptors. *Cell* 1995, 82: 531–533, with permission.

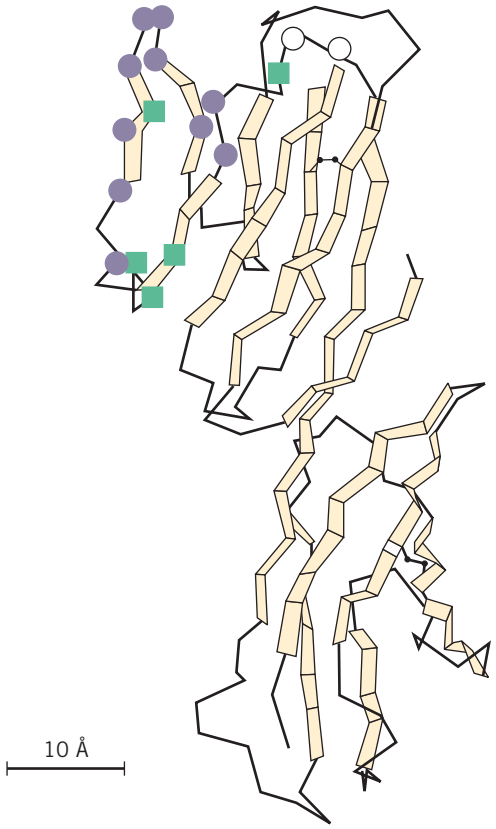

FIGURE 3.4 Molecular structure of the two outermost domains of CD4 to show the site that binds gp120, the VAP of HIV. The filled circles and squares indicate amino acids that are part of the virus spike-binding domain. After Wang J, Wan Y, Garrett TP *et al.* Atomic structure of a fragment of human CD4 containing two immunoglobulin-like domains. *Nature* 1990, 348: 411–419, with permission.

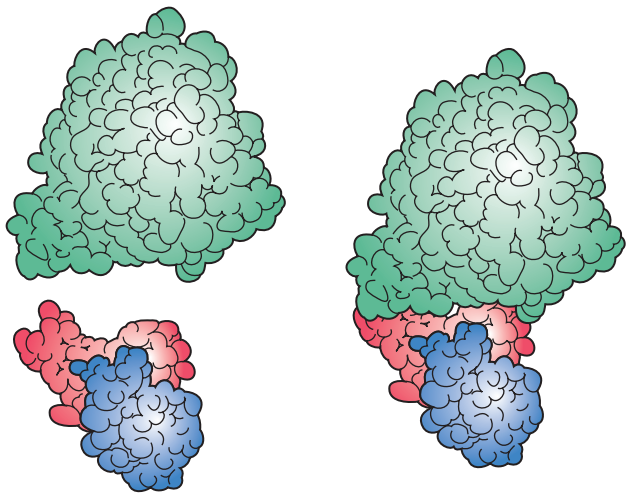

FIGURE 3.5 The interface between a virus attachment protein and its cognate receptor. The spike protein of SARS (severe acute respiratory syndrome) virus attaches to host cells via its cellular receptor, ACE2 (angiotensin-converting enzyme 2). In this space-filling image, the spike ACE2 receptor is shown in green and the receptor binding domain of the spike is shown in red with its underlying core structure in cyan. As this figure illustrates, there is a broad surface where VAP contacts the receptor, although mutational analysis indicates that only a few of the contact residues are critical for attachment. After Li F, Li W, Farzan M, Harrison SC. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* 2005, 309: 1864–1868, with permission.

of some of the branched carbohydrate sidechains of glycosylated proteins. Different influenza hemagglutinins bind preferentially to different terminal sialic acid residues, depending on the linkage of the sialic acid to a proximal galactose or galactosamine molecule in the carbohydrate chain. Thus, human type A influenza viruses bind most avidly to sialic acid α2,3 galactose configurations while equine type A influenza viruses bind best to sialic acid α2,6 galactose. This subtle distinction illustrates the exquisite specificity of the interaction between the viral attachment protein and its cellular receptor.

Influenza virus has a neuroaminidase protein that can cleave the sialic acid residue from the carbohydrate sidechain of the receptor, destroying the ability of cells to bind the virus. Because of this property, the neuraminidase is also called the 'receptor destroying enzyme'. It may appear paradoxical that the virus can destroy its own cellular receptor, but this facilitates the release of newly budded virus from the surface of infected cells.

Non-protein receptors

In addition to proteins, glycolipids and glycosaminoglycans can serve as virus receptors (Figure 3.6). For instance, some isolates of HIV-1 can infect certain neural and intestinal cells that do not express the CD4 glycoprotein. In this case, it appears that galactosylceramide, a glycosphingolipid, serves as an alternative receptor. The virus appears to bind to the galactose moiety on galactosylceramide via the V3 loop on the viral attachment protein, a different domain than that which binds to the major CD4 receptor. Sialic acid, the receptor for influenza viruses, also occurs as part of some complex lipids – as well as glycoproteins – on cell surfaces, and sialylated lipids can also act as influenza virus receptors.

Glycosaminoglycans are sulfated carbohydrate polymers that comprise part of proteoglycans, complex macromolecules composed of proteins and carbohydrates that coat the surface of cells and form the 'ground substance' or intercellular matrix that is found between cells in many tissues. Heparan sulfate, one such glycosaminoglycan, acts as an attachment factor for herpes simplex viruses, although additional cell surface molecules are required for entry of these viruses (described below). Some generalities about viral receptors are summarized in Sidebar 3.1.

Viruses require their cognate receptor

The ultimate proof that a specific cellular molecule is a receptor for an individual virus is the resistance of animals in which the putative receptor has been 'knocked out'. CEACAM1a (carcinoembryonic antigen-like cellular adhesion molecule 1a) is a receptor for mouse hepatitis virus (MHV). MHV is a coronavirus of mice which causes a systemic infection with acute hepatitis. MHV invades the central nervous system, where it infects glial cells leading to acute demyelination and clinical paralysis. CEACAM1a is a cellular adhesion molecule and has a number of other physiological roles, but CEACAM1a$^{-/-}$ (knockout) mice are viable although they have some biochemical abnormalities. Figure 3.7 compares MHV infection in control animals and knockout mice.

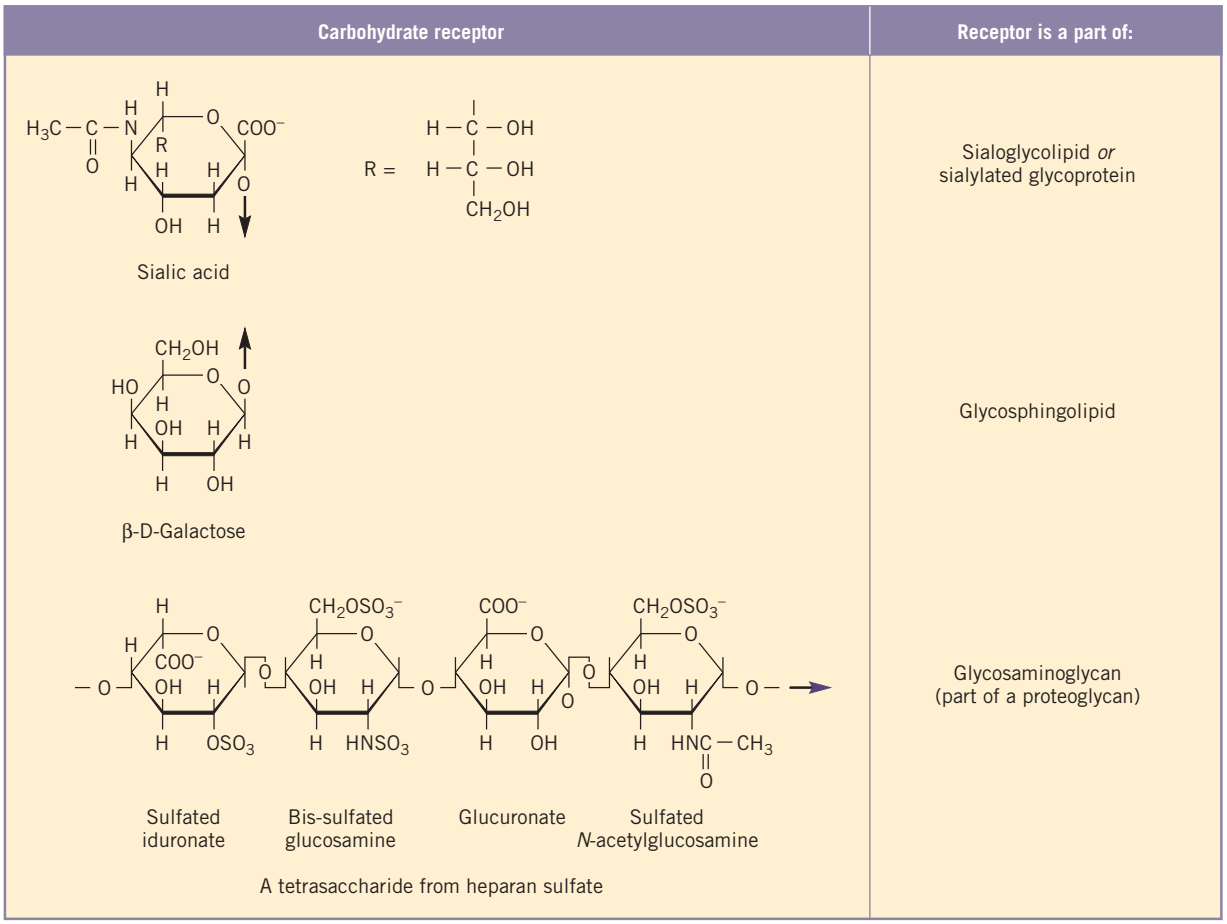

FIGURE 3.6 Some examples of non-protein viral receptors. This diagram illustrates a sialic acid receptor for type A influenza viruses; a galactose receptor (part of a glycosphingolipid, galactosylceramide) that is an alternative receptor for HIV-1; and a glycosaminoglycan (heparan sulfate, part of a proteoglycan) that is a receptor for herpes simplex viruses. The diagrams show only the sugar residues and the arrows indicate where they are bound to the remainder of the molecules of which they are a part. Cognate viral attachment proteins are: HIV-1: the V3 loop on gp120; influenza virus: the distal tip of the HA1 molecule; HSV: the gB or gC glycoprotein. In all instances, the sugar residue is responsible for binding the viral attachment protein and this residue may be part of a glycolipid (galactosylceramide; sialic acid), a glycoprotein (sialic acid) or a complex proteoglycan (heparan sulfate). After Stryer L. *Biochemistry*, WH Freeman, New York, 1988, with permission.

SIDEBAR 3.1

Viral receptors: some principles

- A variety of molecules, including glycoproteins, glycolipids, and glycosaminoglycans, can serve as viral receptors.
- The domain of the receptor that binds the virus may be either a polypeptide sequence or a carbohydrate moiety, often located at the external tip of the receptor molecule.
- Different viruses employ different cellular receptors.
- A given virus isolate may employ several alternative cellular molecules as receptors.
- In some instances, viral entry requires two or more different co-receptors on the cell surface.
- Different isolates of the same virus may prefer different receptors. A specific virus isolate may alter its receptor preference by selection of a mutant VAP during serial passage in animals or cell cultures.
- Not all cells that express the viral receptor are capable of supporting the complete cycle of viral replication.
- Host species differences in the receptor and its orthologs may restrict the host range of a virus.

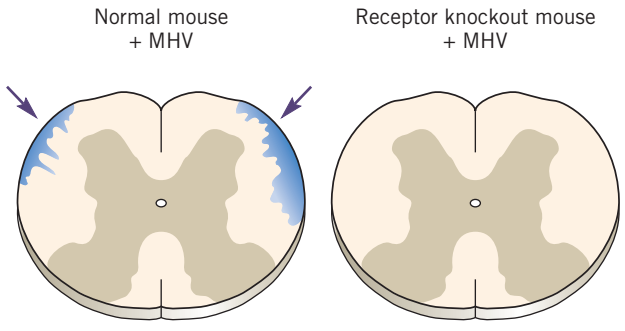

FIGURE 3.7 The cellular receptor is an essential requirement for susceptibility to the cognate virus. A comparison of control mouse (left) with a mouse in which the gene for CEACAM1a (carcinoembryonic antigen cellular adhesion molecule 1a) has been deleted (right). Both mice were infected with mouse hepatitis virus, a coronavirus that causes a late demyelinating disease of the spinal cord. The spinal cord of the control mouse shows severe demyelinating lesions of the ventral white matter (arrows), in contrast to the normal appearance of the spinal cord in the CEACAM1a$^{-/-}$ mouse. The area of demyelination is shown in blue in this false color image. After Hemmila E, Turbide C, Olson M *et al.* CEACAM1a$^{-/-}$ mice are completely resistant to infection by murine coronavirus mouse hepatitis virus A59. *Journal of Virology* 2004, 78: 10156–10165.

Virus and family	Extra cellular virion: stable hydrophilic infectious	First processing step: priming	Transition state: metastable hydrophilic infectious	Second processing step: triggering	Pre-entry virus particle: stable hydrophobic poorly infectious	Third processing step: membrane interaction
Influenza *orthomyxovirus*	HA_0	Proteolytic cleavage	HA_1 + HA_2	Low pH	HA_1* and HA_2*	Fusion
TBE *flavivirus*	prM-E	Proteolytic cleavage	E	High [H+]	E*	Fusion
Polio *picornavirus*	VP0-VP3-VP1	Autolytic cleavage	VP4 + VP2 + VP3 + VP1	Receptor binding	VP4* + VP2* + VP3* + VP1*	Pore formation?
Reo *reovirus*	σ3-μ1	Proteolytic degradation	μ1	High [K+] Autolytic	μ1*	Perforation?

TABLE 3.1 Comparison of entry pathways used by different viruses. Shown are two enveloped viruses (influenza and TBE) and two naked capsid viruses (poliovirus and reovirus) and the table emphasizes some parallels in the entry process used by these quite diverse viruses
HA: hemagglutinin; VP: virus protein; E: envelope protein; prM: pre-matrix protein; *: signifies an activated state of the protein. After Chandran K, Farsetta DL, Nibert ML. Strategy for nonenveloped virus entry: a hydrophobic conformer of the reovirus membrane penetration protein μ1 mediates membrane disruption. *Journal of Virology* 2002, 76: 9920–9933, with permission.

Knockout mice did not support the replication of virus and did not develop demyelinating lesions, indicating that CEACAM1a is the sole receptor for this virus.

Viral attachment proteins

As noted above, attachment of the virus particle to its cellular receptor is conferred by a virion surface protein, often called the viral attachment protein (VAP). As a rule, there is a single VAP, although other viral surface proteins often play an essential role in the steps that following the initial attachment of virions to the cell surface. For enveloped viruses, the VAP is almost always a surface glycoprotein that oligomerizes to form spikes that protrude from the viral envelope. Mutational mapping indicates that a restricted domain on the outermost region of the VAP is responsible for binding, as shown in Figure 3.8 for the influenza virus hemagglutinin. For non-enveloped naked viruses, the VAP is one of the surface proteins that forms the external ordered structure of the viral capsid.

Antibodies that neutralize a virus (see Chapter 6) frequently act by blocking the receptor-binding domain of the VAP. Often the receptor-binding domain of a VAP lies in a recessed pocket where it is not directly accessible to antibodies. In such instances, antibodies that bind to epitopes close to the pocket can neutralize the virus. However, viral mutations that alter the epitope structure can escape neutralization, without affecting receptor binding. Such escape mutants allow many different virus serotypes to use the same receptor-binding domain of the VAP, as seen for rhinoviruses. Neutralizing antibody escape mutants of this kind are important for the persistence of some viruses, including HIV (see Chapters 10 and 14).

Viral entry

Viral entry is a multistep process that follows attachment of the virion to the cellular receptor and results in deposition of the viral genome (nucleocapsid) in the cytosol or in the nucleus. Viruses use a number of different pathways, which are the subject of ongoing research and the molecular details are not yet entirely understood for any of them (Table 3.1).

Influenza virus

The low pH-dependent entry of enveloped viruses is exemplified by influenza virus (Figure 3.8). The sequential steps in entry include: attachment of the hemagglutinin (HA) spike (the VAP) to sialic acid receptors on the cellular surface; internalization of the virion into a clathrin-coated pit, movement into an endocytic vacuole; fusion of the endocytic vacuole with lysosomes, with lowering of the pH below pH 5.5; a drastic alteration in the structure of the HA1 trimer, with re-orientation of the HA2 peptide to insert its hydrophobic fusion peptide into the vacuolar membrane; fusion of viral and vacuolar membranes; and release of the viral nucleocapsid into the cytosol. Nearly all RNA viruses replicate in the cytoplasm, but influenza virus is an exception. Following fusion, the eight influenza genome segments are transported through the nuclear pore, using a nuclear import signal-requiring transport mechanism.

HIV

HIV is another important example in which fusion occurs at the plasma membrane (without internalization of the virion and without the requirement for acidic pH); again, there are steps involving receptor-induced conformational changes in the structure of the VAP (gp120 surface protein of HIV) that leads to fusion of viral and plasma membranes (described in Chapter 14).

Herpes simplex virus (HSV)

The entry of HSV, an α-herpesvirus, is a good example of viral entry that utilizes multiple types of spike glycoproteins to bind to and enter cells. As summarized in Table 3.2, there are at least three distinct events: first, attachment of gB to heparan sulfate, a complex carbohydrate

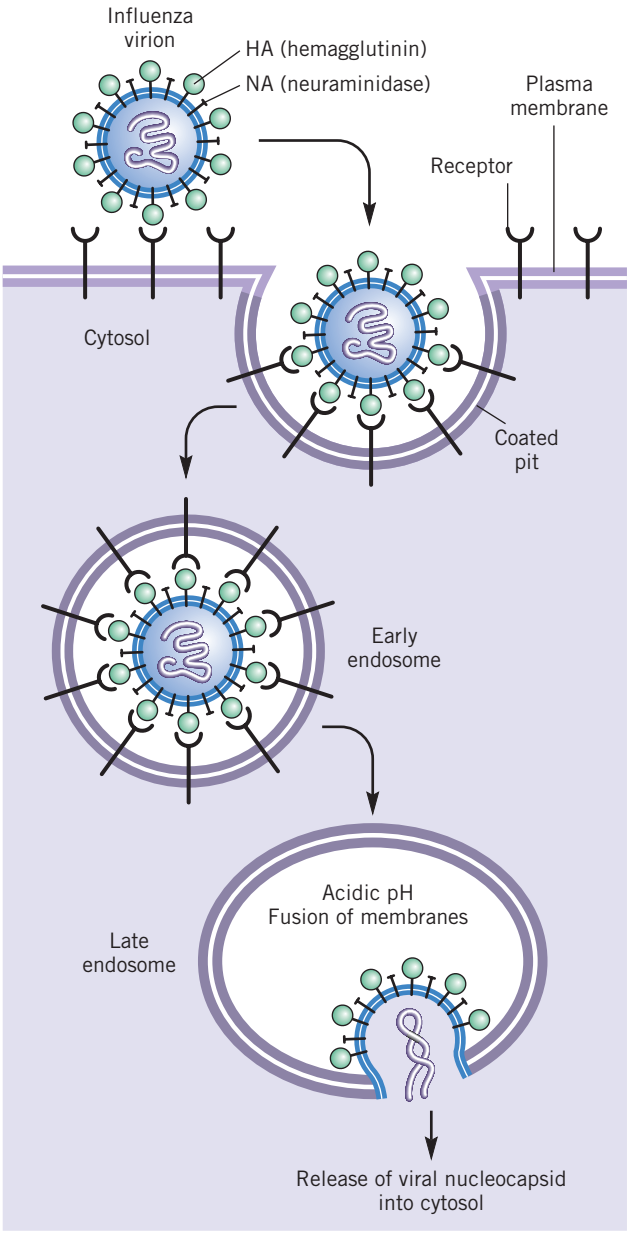

FIGURE 3.8 Diagram of the stepwise entry of influenza virus at a cellular level. Key events are attachment of the virion; internalization of the virion by endocytosis; lowering the pH (to <pH5.5) of the endocytic vacuole leading to drastic reconfiguration of the viral attachment protein (hemagglutinin, HA1 and HA2); insertion of a hydrophobic domain of HA2 into the vacuolar membrane; fusion of the viral and vacuolar membranes; release of the viral nucleocapsid into the cytosol. In the case of influenza virus, there is a further series of steps in which the nucleocapsid complexes are imported into the nucleus, but this is atypical for RNA viruses, most of which replicate in the cytosol. This cartoon shows a nucleocapsid containing one of the eight genome segments of influenza virus.

expressed on the surface of many cell types; second, binding to one of several potential protein receptors on the cell surface; and third, fusion of the viral envelope with the plasma membrane. Tissue tropism is determined in part by the distribution of the protein receptors: both HVEM (herpesvirus entry mediator) and nectins are expressed on epithelial cells and fibroblasts (cells initially infected),

Step in entry	Viral proteins	Cellular targets	Comment
Initial binding	gB or gC	Heparan sulfate (ubiquitous)	Can be mediated by alternate viral glycoproteins, not absolutely required for infection
Further attachment	gD	HVEM (lymphocytes, epithelial cells, fibroblasts) or nectin-1 or nectin-2 (neurons, epithelial cells, fibroblasts)	Alternate receptors used for different cell types
Fusion	gD + gB + gH–gL	Plasma membrane	Requires all listed viral glycoproteins

TABLE 3.2 Viral and cellular molecules involved in the entry of an α-herpesvirus. HSV entry is mediated by several viral glycoproteins that participate in sequential steps leading to fusion of the HSV envelope with the plasma membrane and the entry of the viral capsid into the cytosol
Heparan sulfate: a glycosaminoglycan; HVEM: herpesvirus entry mediator, member of the TNF receptor family of proteins; nectins: members of the immunoglobulin superfamily of proteins. After Spear PG. Herpes simplex virus: receptors and ligands for cell entry. *Cellular Microbiology* 2004, 6: 401–410, with permission.

while HVEM is likely the receptor on lymphocytes and nectins are the receptors on neurons (secondarily infected cells). Thus, HSV uses at least two different receptors to infect different cell types. HSV can also use an endocytic entry pathway in some types of cultured cells.

Naked capsid viruses

For naked capsid non-enveloped viruses, viral entry also begins with attachment to the cellular receptor and ends with release of the viral nucleocapsid or the genome into the cytosol or nucleus. However, there are fundamental differences from enveloped viruses, since membrane-membrane fusion is not involved. Figure 3.9 shows one example, SV40 (simian virus 40), a polyoma virus. SV40 is a small (45 nm diameter) viral capsid with a double-stranded (ds) DNA genome. It binds to sialic acid receptors, enters via cellular caveolae, is transported along microtubules, enters the endoplasmic reticulum and crosses the nuclear membrane through NPCs (nuclear pore complexes).

Most DNA viruses, with the exception of poxviruses, replicate within the nucleus of the cell. To initiate replication, the nucleocapsid must be transported through the cytosol to the nuclear membrane, cross via nuclear pores and enter the nucleus. The steps in transmission of the nucleocapsid into the nucleus involve specific transport mechanisms, and there are a number of different strategies that are used to import the viral genome (and associated proteins) through the NPC (nuclear pore complex).

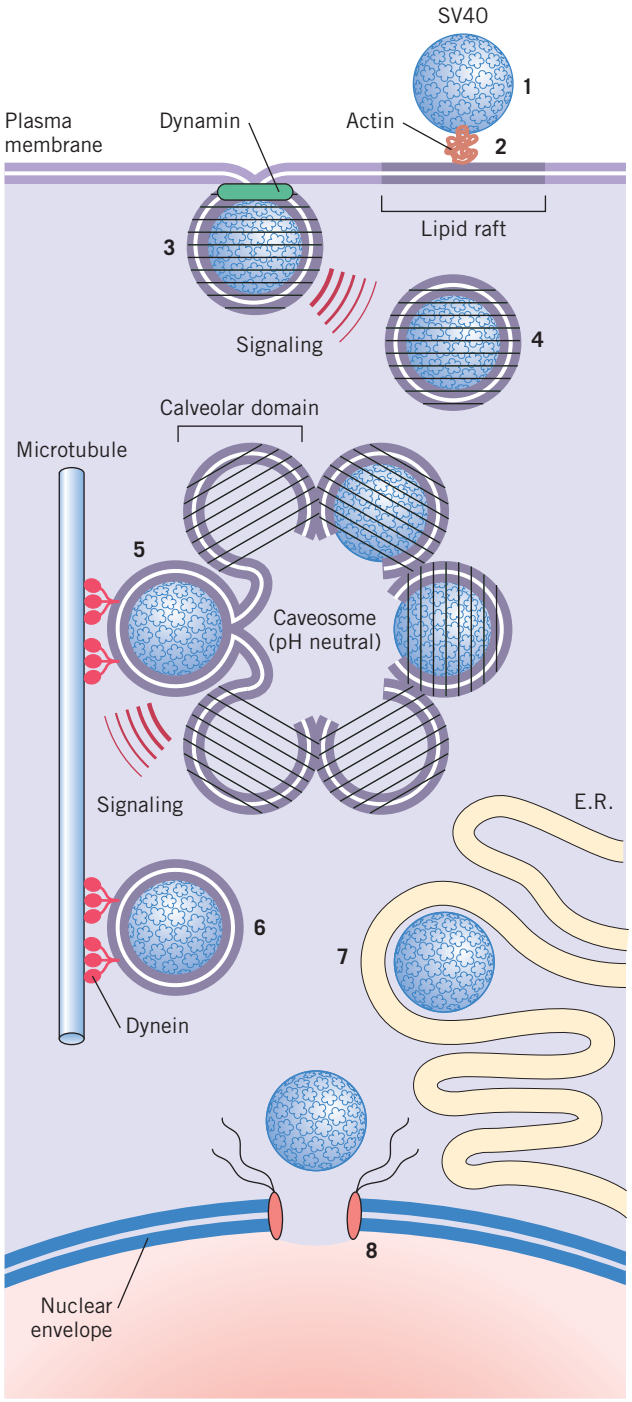

FIGURE 3.9 Entry of a naked capsid virus. Steps in entry for SV40 (simian virus 40) include: (1) virus binds to gangliosides in the plasma membrane; (2) virus is inserted into lipid rafts; (3) lipid rafts are incorporated in caveolae; (4) caveolar endocytosis occurs; (5) caveolae associate with caveosomes and virus enters caveolin-free vesicles; (6) vesicles associate with dynein and are carried along microtubules; (7) vesicles pass virus to the smooth endoplasmic reticulum; (8) virus is released close to nuclear pore complexes (NPCs) and enters the nucleus. After Smith AE, Helenius A. How viruses enter cells. *Science* 2004, 304: 237–242, with permission.

TROPISM

Tropism determined by cellular receptors

As indicated at the beginning of this chapter, cellular receptors are unequally expressed on different cell types and this distribution limits the cell types that are permissive for a given virus group. One classic example is the poliovirus receptor.

Poliovirus

Poliovirus was one of the first animal viruses to be isolated (1908) and it was early recognized that the virus could only infect humans and non-human primates, in contrast to many other human viruses like influenza virus and herpes simplex virus that could readily infect rodents and other experimental animals. Two findings focused attention on the viral receptor as a determinant of the species specificity of poliovirus: homogenates of primate tissues could bind virus much more avidly than similar homogenates from non-primates and transfection of RNA extracted from virions could produce a single round of infection in non-primate cells. Taken together, these observations suggested that the species specificity (or tropism) of poliovirus was determined by the viral receptor. It was inferred that the species restriction of poliovirus is due to the close similarities between the receptor glycoprotein expressed by all primates, while the homologous glycoprotein differs significantly in non-primate species. Cytogenetic experiments indicated that the putative poliovirus receptor was encoded by human chromosome 19.

In 1989, using DNA transfection, the gene encoding the human poliovirus receptor (PVR) was isolated and shown to encode a previously unknown surface protein of the immunoglobulin superfamily. Poliovirus is an enterovirus that replicates in the gut and is excreted in the feces. It produces a viremia, invades the CNS and then infects and destroys the anterior horn cells, a very specific set of lower motor neurons in the medulla and spinal cord, thereby producing a characteristic flaccid paralysis (see Figure 2.1). A survey of primate tissues indicated that mRNA for the poliovirus receptor was expressed at differing levels in many organs. A high level of expression in anterior horn cells was consistent with their susceptibility to poliovirus infection. However, there were many discordances between receptor expression and viral replication. Table 3.3 shows that certain tissues or cells which expressed the receptor mRNA robustly were not infected while other cells that expressed only modest levels of receptor were infected. It appeared that receptor expression is necessary for susceptibility to infection, but is not sufficient to explain the restricted tissue and cellular replication of poliovirus.

To explore the role of the PVR, transgenic mice were created that express the human PVR (TgPVR). These mice could be infected by intracerebral, intramuscular, intraperitoneal and intranasal routes of inoculation, following which they developed infection of the brain and spinal cord and became paralyzed. However, poliovirus infection of TgPVR mice does not completely duplicate infection in primates, since these mice cannot be infected by the enteric route. Again, it appears that the PVR is necessary but not sufficient completely to reproduce primate susceptibility.

A line of TgPVR mice was created in which the gene for the receptor for IFNα/β was deleted, and these mice

Tissue	Cells	PVR mRNA expression	Poliovirus replication	Cellular destruction
Central nervous system	Neurons			
	Anterior horn spinal cord	High	High	Severe
	Posterior horn spinal cord	High	High	Minimal
	Medulla	High	High	Moderate
	Cerebellum	High	Moderate	Minimal
	Midbrain	High	Moderate	Minimal
	Forebrain	High	Moderate	Minimal
Thymus	T lymphocytes	High	None	None
Kidney	Epithelial, tubule cells	High	None	None
Lung	Alveolar cells	High	None	None
Adrenal	Endocrine cells	High	None	None
Intestine	Many	Low	None	None
Spleen	Lymphocytes	Low	None	None
Skeletal muscle	Myocytes	Low	Moderate	None

TABLE 3.3 Receptor expression is necessary but not sufficient to explain poliovirus tissue tropism. Expression of mRNA for the human poliovirus receptor (PVR) in transgenic mice and expression of poliovirus RNA and cellular damage following infection of the same mice. Virus replication is confined to cells where the PVR is expressed at a high level, but there are many tissues that are not infected in spite of PVR expression
After Ren R, Racaniello VR. Human poliovirus receptor gene expression and poliovirus tissue tropism in transgenic mice. *Journal of Virology* 1992, 66: 296–304, with permission.

were compared with interferon receptor-competent TgPVR. After infection with poliovirus, the virus replicated to high titers in the central nervous system in both kinds of mice, but in the TgPVR/IFNαRKO mice, virus also replicated in many other tissues expressing the PVR (Table 3.4). In addition, the TgPVR/IFNαRKO could be infected by the oral route with a high dose of virus, while the TgPVR were not infected by the oral route. It was found that the poliovirus resistant tissues expressed constitutive levels of IFN receptors – in contrast to the nervous system – and it was suggested that this host innate response (see Chapter 5) could be a critical factor that explained the restricted tissue tropism seen in TgPVR mice.

Tissues	Virus titer (log10 PFU per g tissue) in three strains of mice		
	Control	TgPVR	TgPVR/IfnarKO
Spinal cord	<2	8.3	8.3
Brain	3.4	6.8	7.3
Lung	3.0	3.6	6.8
Liver	2.5	3.0	6.7
Kidney	3.5	3.3	7.2
Spleen	4.6	5.0	7.3

TABLE 3.4 The effect of IFN receptors upon the tropism of human poliovirus in mice. Three strains of mice – controls, TgPVR and TgPVR/IFNαRKO – were infected intravenously with $10^{7.3}$ PFU of Mahoney strain of human poliovirus and tissues were assayed 3 days after infection. Poliovirus replicates to high titer in the central nervous system of both strains of TgPVR mice, but has a broad tissue tropism only in the interferon receptor knockout mice. Titers of $<10^4$ likely represent residual injected virus, since the virus does not replicate at all in control animals
TgPVR: transgenic mice expressing the poliovirus receptor; TgPVR/IFNαRKO: poliovirus receptor mice in which the gene for the receptor for interferon α/β was knocked out.
After Ida-Hosonuma M, Iwasaki T, Yoshikawa T *et al.* The alpha/beta interferon response controls tissue tropism and pathogenicity of poliovirus. *Journal of Virology* 2005, 79: 4460–4469.

Parvovirus B19

Parvovirus B19 causes a mild exanthem (known as 'fifth' disease or exanthem subitum) in children, but is mainly of clinical importance because of its predilection for erythrocyte precursors in the bone marrow. This ability to replicate in and destroy precursors of red cells, which can cause a severe anemia, is an unusual tropism exhibited by very few viruses. Autonomous parvoviruses such as B19 are restricted in their replication to dividing cells, since some of the cellular enzymes required for replication of parvoviral DNA are only expressed during the S phase of cell division. The receptor for B19 virus has been identified as globoside, a neutral glycosphingolipid and the receptor domain is probably the terminal galactose residue. Tissue surveys show that globoside is present on many tissues of mesodermal origin but comprises a higher proportion of the glycosphingolipids on erythrocytes and their rapidly dividing precursors than on other tissues. In this instance, tropism is apparently associated with robust expression of the viral receptor plus the restriction of

replication to rapidly dividing cell populations. Some humans lack the receptor for B19 virus and their cells are totally resistant to infection. Again, receptor expression is necessary but is not the only determinant of tropism.

Lymphocytic choriomeningitis virus (LCMV)

Different strains of LCMV exhibit differences in their tissue tropism in vivo; clone 13 replicates preferentially in the liver and spleen while the Armstrong strain replicates best in the brain. Furthermore, these differences are determined by a single amino acid at position 260 in the viral glycoprotein (the viral attachment protein), implying that tropism may be correlated with ability to bind to the viral receptor. Identification of the receptor as α-dystroglycan has led to the recognition that differences in the degree of affinity to this single receptor appear to correlate with differential tropism. Thus, LCMV strains with high affinity for the receptor replicate preferentially in the white pulp of the spleen, ablate the virus-specific T lymphocyte cell response and initiate a persistent infection. Virus strains with a low affinity for the receptor replicate preferentially in the red pulp of the spleen, do not ablate the immune response and are rapidly cleared. This example underlines the important nuance that differences in the quantitative interaction of a virus with a single receptor may have significant influence upon tropism and the outcome of an infection.

SIDEBAR 3.2

Some determinants of viral tropism

- Cellular receptors
- Activation of viral attachment or fusion proteins by host proteases
- Temperature sensitivity of virion
- pH lability of virion
- Cellular transcription factors
- Anatomical barriers
- Host innate and acquired immune response

Other determinants of tropism

In addition to receptor specificity, there are a number of other determinants of tropism, some of which are summarized in Sidebar 3.2. As a general rule, viruses with an extended cellular host range spread more widely in vivo and cause more serious disease.

Cellular protease requirement

A number of enveloped viruses are not infectious when they bud from cells as mature virions because one of the viral surface glycoproteins (either the viral hemagglutinin or the viral fusion protein) requires proteolytic cleavage at a particular site to be activated (Figure 3.10). In such instances, infectious virus is only produced by replication in cell types that secrete a cellular protease into the endoplasmic reticulum or Golgi apparatus, through which the viral envelope proteins are processed. Alternatively, some viral fusion glycoproteins may be cleaved by enzymes in extracellular fluid. In many instances, the degree of susceptibility to proteolytic cleavage is determined by a few amino acids (such as one versus several arginines) at the site of cleavage, so that mutations in one or two critical amino acids can alter the tissue tropism.

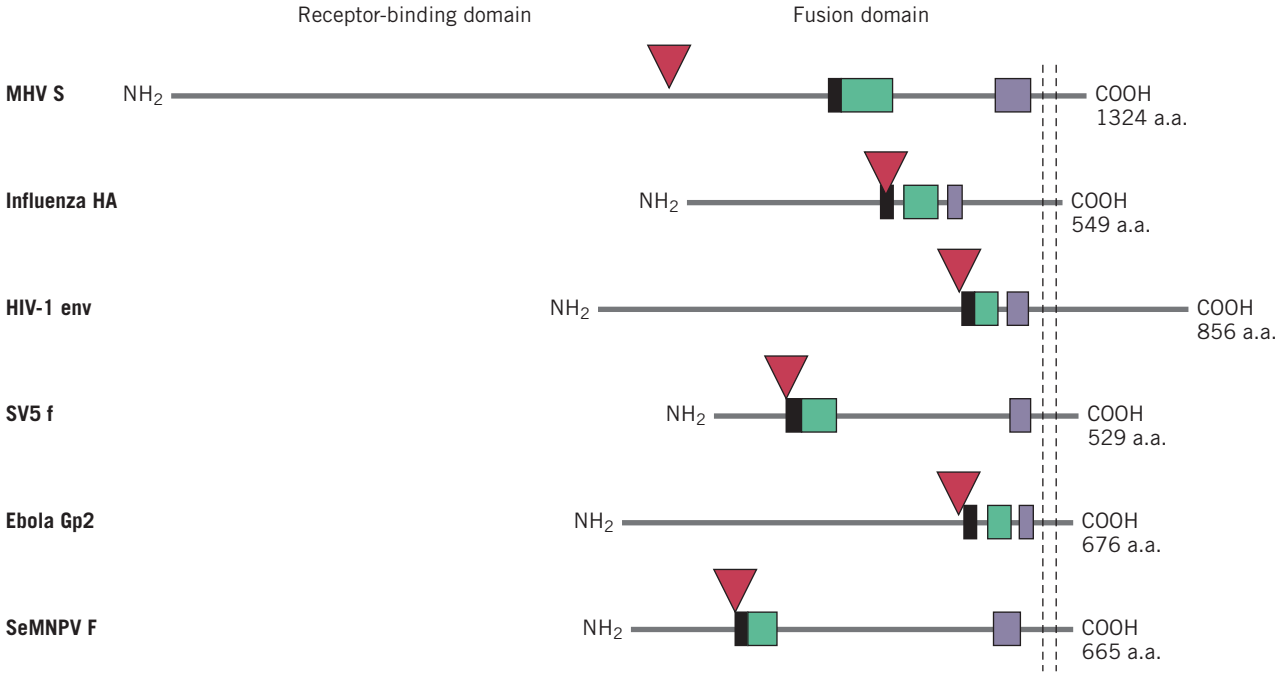

FIGURE 3.10 Schematic representation of the type 1 viral fusion proteins of six different virus families. The proteins are oriented with the exodomain pointing to the left, the transmembrane domain indicated by vertical broken line and the endodomains to the right. Red triangles indicate the cleavage sites, black boxes the putative fusion peptide and the vertical and horizontal bars HR1 and HR2 (heptad repeats), respectively. MHV: mouse hepatitis virus; SV5: simian virus 5; Ebola Gp2: ebola virus glycoprotein 2; SeMNPV F: S exigua multicapsid nucleopolyhedrosis virus fusion protein. After Bosch BJ, van der Zee R, de Haan CAM, Rottier PJM. The coronavirus spike protein as a Class I virus fusion protein: structural and functional characterization of the fusion core complex. *Journal of Virology* 2003, 77: 8801–8811, with permission.

The paramyxoviruses are enveloped viruses that carry two surface glycoproteins, the hemagglutinin-neuraminidase (HN) that acts as the viral attachment protein and binds to the sialic acid containing receptors, and the fusion (F) protein, that mediates fusion between the viral envelope and the plasma membrane. In many cases, the fusion protein exists as an inactive F_0 precursor. Conversion into the fusion-active F_1 form requires proteolytic cleavage of the F_0 protein in the intra- or extracellular milieu. Not all cells secrete the activating proteases, and this limits virus replication to selected cell types, and explains why some paramyxoviruses do not spread beyond the respiratory tract.

A case in point is Newcastle disease virus, a paramyxovirus of birds. Virulent isolates of Newcastle disease virus encode a fusion protein that is readily cleaved by furin, a proteolytic enzyme present in the Golgi apparatus, so that the protein is activated during maturation prior to reaching the cell surface, and before budding of nascent virions. This makes it possible for virulent strains of the virus to infect many avian cell types, thereby increasing its tissue host range, and causing systemic infections that are often lethal. In contrast, avirulent strains of Newcastle virus encode a variant fusion protein that is not cleaved during maturation in the Golgi, so that nascent virus requires activation by an extracellular protease. The required protease is found only in the respiratory or enteric tracts, thereby limiting tropism to surface cells and conferring an attenuated phenotype on the virus.

Temperature of replication

Most human viruses replicate optimally at 37°C, which is the core body temperature of humans. However, some mucosal surfaces such as the upper respiratory tract have a lower temperature, about 33°C. Certain viruses, such as rhinoviruses, that replicate in the epithelial cells of the nose and throat, have been selected to grow optimally at 33°C. Rhinoviruses replicate poorly at 37°C and were originally isolated only when cell cultures were maintained at 33°C. Such viruses are usually restricted in their tissue distribution by their relative inability to replicate at 37°C, which limits their spread beyond the upper respiratory tract.

Acid lability and protease digestion

A number of viruses are enteric, initiate infection by ingestion, and are excreted in the feces, to be transmitted by the fecal-oral route. The gastrointestinal tract presents a harsh environment, due to the acid pH of the stomach, the alkaline pH of the intestine, and the destructive effects of pancreatic digestive enzymes. In general, enterotropism is limited to viruses that can survive these adverse conditions, although there are some exceptions. Thus, although most respiratory viruses are swallowed to some extent, only a few are enterotropic and can survive and replicate in the gastrointestinal tract; adenovirus is an example. Some enterotropic viruses, such as reoviruses, have exploited the adverse conditions of the enteric tract so that they undergo conversion to an infectious subvirion particle (ISVP) by digestive enzymes. Proteolytic cleavage leads to a conformational change in the viral attachment protein, σ1, permitting the virions to bind to a receptor on microfold (M) cells, the first step in reoviral entry in the gut.

Transcriptional control of tropism

Following uncoating and entry of the viral genome, there is a complex program of replication, in which the virus utilizes a number of host proteins to transcribe its genome, translate virus-encoded messages, and assemble and release new infectious virions. Since cell types differ in their ability to support each of these multiple steps, tropism can be determined by cell-specific differences in replication.

One example is the papillomaviruses, DNA viruses that replicate in skin and may cause tumors, varying from benign warts to malignant cancer of the cervix (Figure 3.11). The skin is a somewhat unique tissue, in which new cells are continuously produced at the innermost basal layer of the epidermis, where germinal cells divide. These cells gradually move outward, differentiating into keratinocytes that are constantly being sloughed from the superficial layers of the skin. Papillomaviruses commence their replication in germinal cells that are permissive for replication of the viral genomes. However, germinal cells produce proteins that block the transcription of late structural genes of the virus. As the infected basal cells move outward and begin to differentiate, they become permissive for transcription and translation of the papillomavirus structural genes, so that complete infectious virus is only formed in cells that are about to be sloughed. Release of virus from the superficial layers of dead cells promotes the transmission of infection to new sites on the infected person and also to new uninfected hosts. Not surprisingly, only the skin (and mucous membranes for some papillomaviruses) provides the complex cascade of transcription factors that can support the complete program of viral replication, explaining the very specialized tropism of papillomaviruses. Papilloma virus receptors are not sufficient for infection, since they are expressed on many non-dermal cell types where virus cannot replicate.

Retroviruses are RNA viruses that have a complex life cycle involving transcription of their genome into a DNA transcript; this transcript is imported into the nucleus where it dictates both the replication of nascent RNA genomes and the transcription of viral messenger RNA (discussed in Chapter 11). Promoters and enhancers that regulate these transcriptional events are located in the 'long terminal repeat' or LTR found at both ends of the integrated viral genome. Some avian retroviruses (avian leukosis sarcoma viruses) are oncogenic, but various members of the group differ in the location and nature of the tumors that they induce, reflecting variation in tissue tropism. Using molecular methods to construct genetic hybrids between viruses with different patterns of tropism, these differences in tropism have been mapped in part to the LTR (Table 3.5). In this instance viral tropism is partially determined by cellular transcription factors that bind the viral LTR.

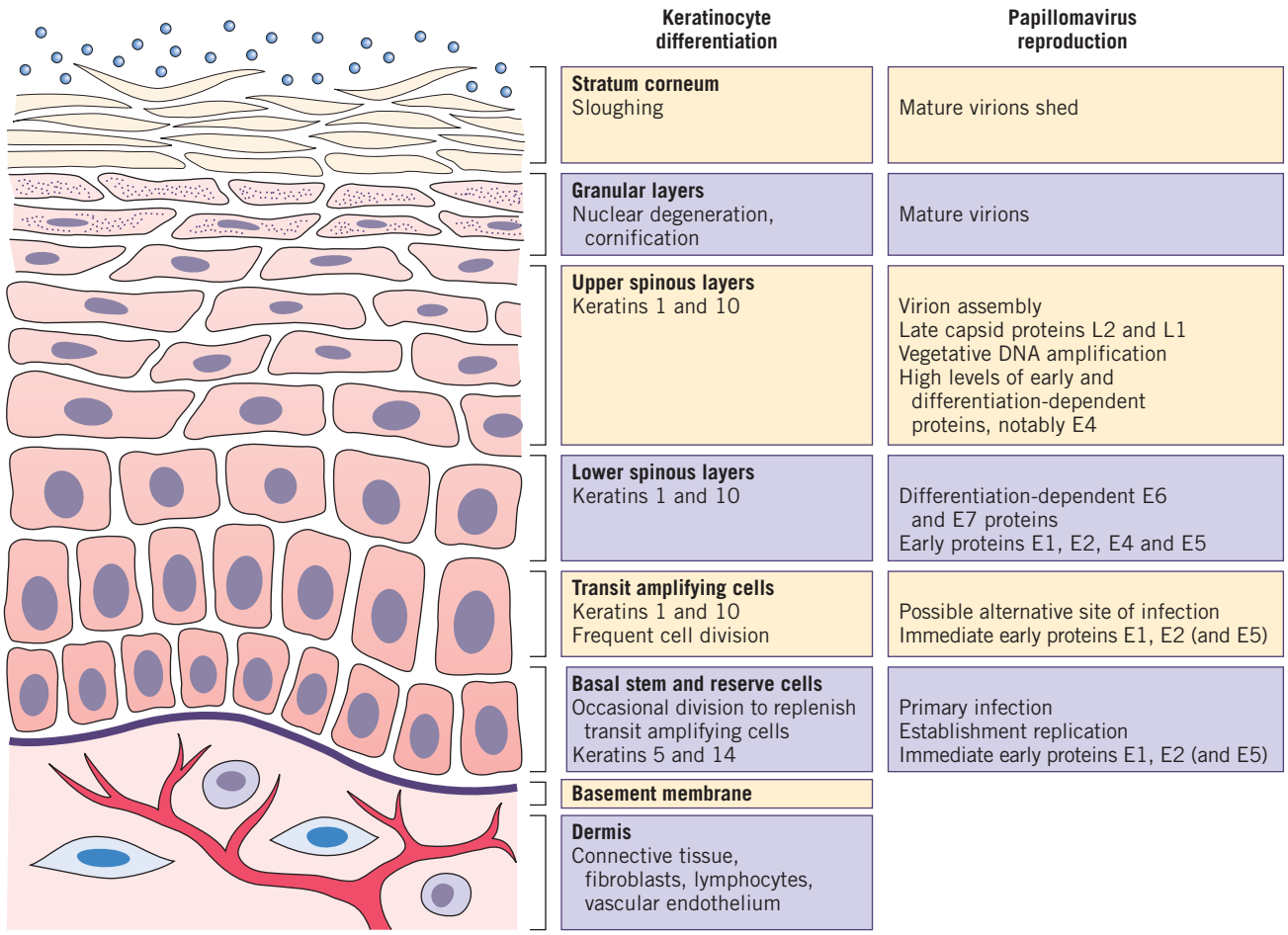

FIGURE 3.11 Sequence of transcriptional events in the synthesis of papillomavirus in different layers of the epidermis. This diagram shows the steps in generation and maturation of keratinocytes and the corresponding steps in viral replication. After Chow LT, Broker TR. Small DNA tumor viruses, in Nathanson N *et al.* (eds) *Viral pathogenesis*, Lippincott Raven Publishers, Philadelphia, 1997, 267–301, with permission.

Env	LTR	Replication	
		Muscle	Bursa
A	RAV-1	++++	++++
A	RAV-0	–	++++
E	RAV-1	–	++++
E	RAV-0	–	++++

TABLE 3.5 Tropism of avian leukosis virus for muscle and bursal tissues of chickens is determined by the viral envelope and by the viral LTR (long terminal repeat). Replication in muscle appears to require the A envelope and also the RAV-1 LTR, illustrating the multifactorial effect of viral genes on tropism

Env: the genotype of the envelope protein that serves as the viral attachment protein for avian leukosis virus; LTR: the LTR was derived from either of two viruses, RAV-1 (Rous associated virus) or RAV-0. After Brown DW, Robinson HL. Influence of env and long terminal repeat sequences on the tissue tropism of avian leukosis viruses. *Journal of Virology* 1988, 62: 4828–4831, with permission.

Anatomic barriers

Another determinant of tropism is the anatomic barriers that limit the passive diffusion of cells and molecules. The ability of a virus to breach these barriers, particularly the blood–tissue barrier, influences its distribution (See Figure 2.7). Neurotropic viruses, which usually cross the blood–brain barrier to enter the central nervous system, illustrate this point. Under controlled experimental conditions, different isolates of poliovirus cause paralysis at different frequencies, and this correlates with the titer of viremia that they induce; presumably high titer viremia increases the probability that poliovirus can invade the central nervous system. Furthermore, the permeability of the blood–brain barrier can be altered in various ways, which influences the frequency of invasion of the central nervous system.

Before the introduction of poliovirus vaccines, it was well established that only about 1% of infections resulted in paralytic disease. Every effort was made to determine and reduce the risk that infection would lead to paralysis. Epidemiological studies indicated that routine childhood injections (such as injected vaccines) were associated with an increased frequency of paralytic poliomyelitis within one month of injection. One hallmark of 'provocation' poliomyelitis was localization to the arm in which the vaccine had been injected. Apparently, intramuscular injection caused a temporary localized increase in permeability of the blood–brain

barrier, increasing the probability of localized virus invasion of the central nervous system.

Host response to infection

As described in Chapters 5 and 6, innate and acquired immune responses have a major impact on the extent and outcome of infection. In addition, tissue-specific responses may vary, and these could influence tropism.

Poxviruses are an important example of this phenomenon, partly because their tropism is apparently not receptor mediated. In addition, with their large genomes, poxviruses have the capacity to encode a variety of genes that help them to evade the host response to infection. Myxoma virus, a poxvirus of rabbits, has long been known to have a host range restricted to rabbits, but the mechanism has been obscure. Recent experiments have shown that blockade of the type 1 interferon (IFN) response renders mouse cells permissive for myxoma virus, and makes mice susceptible to lethal infection with myxoma virus. Studies of several poxviruses have established the importance of differential evasion of host specific antiviral defenses as mechanism of tropism for this group of agents.

Tropism and viral variation

Variation in the pathogenicity or virulence of different isolates of a single virus is an important topic that is the subject of Chapter 9. However, it is appropriate to mention a few examples in which pathogenicity can be associated with variation in tropism.

One striking example is avian influenza virus. A particular serotype (known by its hemagglutinin and neuraminidase as H5N2) circulates in domestic fowl as a relatively innocuous cause of respiratory infection. However, on several occasions, a virulent mutant of this virus has appeared, and caused pandemic fatal disease in commercial poultry flocks, first in Pennsylvania in 1983, subsequently in Mexico in 1994, and in other countries since then. In these epidemics, the hemagglutinin of the virus acquired a mutation – deleting an O-linked glycosylation site or increasing the number of basic amino acids close to the HA1/HA2 cleavage site – that renders the viral hemagglutinin more susceptible to proteolytic cleavage. As a consequence the mutated virus has a much broader tropism, since it can multiply in a wider range of host tissues and replicate to higher titers, converting a mild, sublethal infection to a rapidly fatal disease.

Another example is the differences in pathogenesis between two similar strains of SHIV (chimeric viruses bearing the envelope of HIV inserted into the genetic backbone of SIV, simian immunodeficiency virus) that use different co-receptors (Figure 3.12). When SHIVs are constructed bearing the two different types of HIV gp120 envelope genes, there is a striking difference in pathogenesis (Figure 3.12). The macrophage-tropic SHIV preferentially infects and destroys T cells in the lamina propria of the intestine with little reduction of CD4-positive T lymphocytes in the blood. The T lymphocyte tropic SHIV destroys T cells in the regional lymph nodes and cause a marked depletion of CD4-positive T lymphocytes in the blood, and the rapid onset of AIDS ('crash and burn' model).

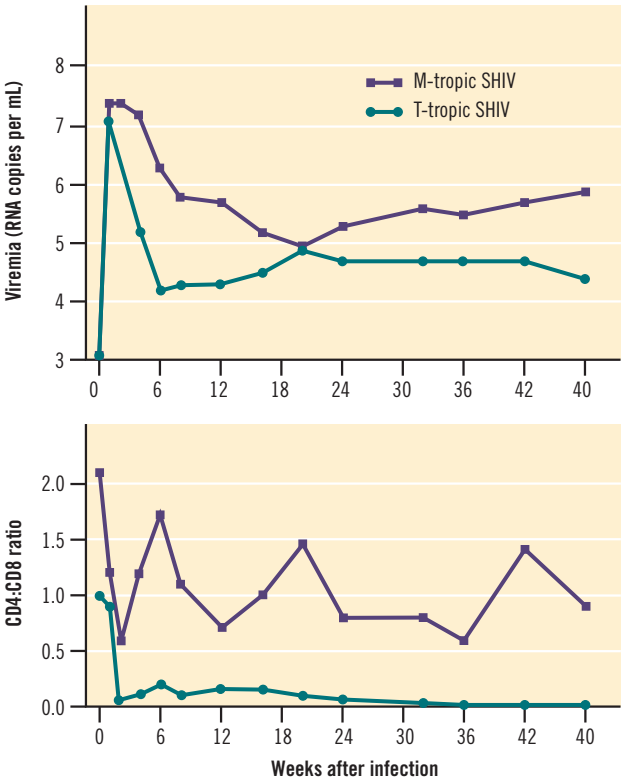

FIGURE 3.12 Differences in the coreceptor specificity of two virus strains influence their tropism. This figure compares two strains of SHIV (chimeric simian human immunodeficiency virus), T-tropic and M-tropic (T cell line-tropic and macrophage-tropic). Upper panel: both viruses produced similar levels of viremia. Lower panel: T-tropic SHIV reduced circulating CD4 T lymphocytes, while M-tropic SHIV did not. After Harouse JM, Gettle A, Tan RCH, Blanchard J, Cheng-Mayer C. Distinct pathogenic sequelae in rhesus macaques infected with CCR5 or CXCR4-tropic strains of SHIV. *Science* 1999, 284: 816–819, with permission.

REPRISE

Tropism refers to the localization of a virus or disease to specific cells and tissues, which has an important influence on its disease signature. The most significant determinants of viral tropism are cellular receptors, which can be proteins, sugars, or lipids. Receptors are necessary – but not sufficient–to confer susceptibility on cells and tissues and some viruses can utilize more than one molecule as a receptor. Following initial virus attachment to a cell, there is a multistep process of entry, followed by replication, assembly, and exit, and each of these steps can also limit the spectrum of susceptible cells. Other factors that determine tropism include the ability of the virus to survive in the hostile extracellular environment, its ability to replicate at different temperatures, its requirement for extracellular enzymes, and its ability to cross the hosts' external and internal anatomical barriers. Finally, cells of different tissues and species differ in their response to infection with a given virus, and this also influences the host range and disease signature of each virus.

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Chapter 4

Virus–Cell Interactions

Neal Nathanson and Diane E. Griffin

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REPRISE

FURTHER READING

HOW DO VIRUSES AFFECT CELLS?

Viral infection of the cell, with subsequent production of infectious virus, often results in cell death. However, there are many variations on this theme. Some viruses can cause highly productive infections without death of the cell and release of progeny virus may occur without cytolysis. Conversely, viruses can induce marked effects by binding to the cell surface without entering the cytosol, or as the result of an incomplete cycle of replication that fails to produce new infectious virions. Alterations in the host cell can be mediated by interfering with a wide variety of normal cell functions, or by inducing a new cascade of cellular processes that leads to apoptosis or programmed cell death. This chapter describes this plethora of virus–cell interactions, many of which can best be studied in cell culture systems (Sidebar 4.1).

SIDEBAR 4.1

Viral effects on host cells: some generalizations

- Viral infection may kill host cells but cytopathic effects are not a necessary consequence of viral replication. Conversely, cytopathic effects can be induced by viruses without completing a productive cycle of replication.
- Binding of a viral attachment protein to its cognate cellular receptor, in itself, can initiate dramatic effects such as cell-to-cell fusion, apoptosis or signaling cascades.
- During infection, some viruses induce a global downregulation of transcription of host genes, while viral genes are actively transcribed. Infection with other viruses may have a differential effect upon transcription of genes of the host cell, some of which are upregulated, others downregulated and some unchanged.
- Virus infection can reduce translation of host mRNA while viral messages are translated at a high rate, due to differential mechanisms of translation initiation.
- Viral infection can alter the expression of certain cell surface proteins, such as the cellular receptor for the virus or MHC (major histocompatibility complex) class I proteins.

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Productive viral replication may not destroy host cells

Does viral replication necessarily compromise normal cellular function? Most viruses utilize only a small fraction of the total protein synthetic machinery of the host cell, often estimated at ~1%. During a highly productive infection, a cell may produce ~1000 virions during a 24-hour period, of which perhaps 100 are infectious. If each virion contains <5000 molecules of protein, then <5 million molecules of viral proteins will be produced daily. This is dwarfed by the daily cellular production of approximately one billion protein molecules, implying that productive viral infection need not compromise the normal synthetic activity of the cell.

Can nascent virus be released without lysis of the host cell? Non-enveloped viruses assemble intracellularly and efficient release often requires lysis of the host cell to free individual virions. However, enveloped viruses, particularly enveloped RNA viruses, normally mature by budding across a cellular membrane, either the plasma membrane or an internal membrane, such as the endoplasmic reticulum or Golgi apparatus. Virions are then released from the plasma membrane or by exocytosis of the content of intracytoplasmic vesicles, in a manner similar to the release of secreted proteins. Under these circumstances, virus dissemination does not depend upon cytolysis and the infected cell may continue to produce infectious virions indefinitely, unless the immune response intervenes to destroy infected cells or limit virus production. Figure 4.1 compares intracellular virus accumulation for a typical cytolytic non-enveloped and a non-cytolytic enveloped virus.

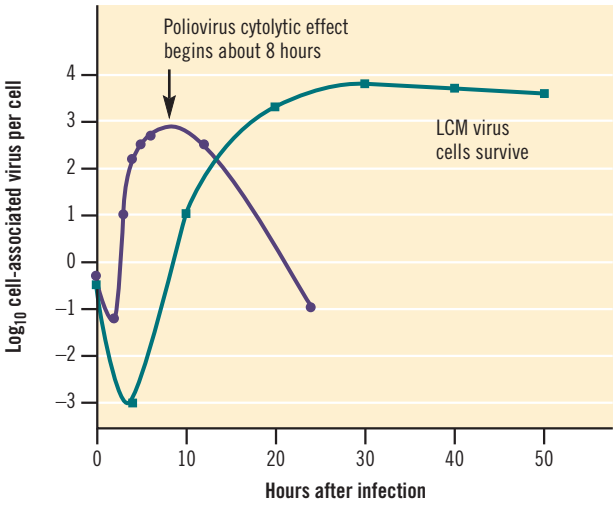

FIGURE 4.1 Comparison of replication of a cytolytic and a non-cytolytic virus. Cytolytic poliovirus replicates rapidly, cells begin to die about 8 hours after infection, terminating virus replication and releasing cell-associated virus. Non-cytolytic lymphocytic choriomeningitis virus (LCMV) replicates more slowly than poliovirus but does not kill most infected cells which continue to produce cell-associated virus, much of which is released by budding through the plasma membrane. Poliovirus: after Darnell JE, Levintow L, Thoren MM, Hooper JL. The time course of synthesis of poliovirus RNA. *Virology* 1961, 13: 271–279. LCMV: Lehmann-Grube F, Popescu M, Shafer H, Geschwinder HH. LCM virus replication in vitro. *Bulletin WHO*, 1975, 52: 443–456, with permission.

Productive viral replication is not required for cytopathology

Conversely, a virus may initiate pathological processes in potential host cells without completing a productive cycle of infection. From the first contact, a virus may impact the potential host cell. Fusion initiated by enveloped viruses is a most dramatic example. As described in Chapter 3, fusion is a critical step in the cellular entry of enveloped viruses and leads to deposition of the viral nucleocapsid complex in the cytosol. When applied to uninfected cells, a viral inoculum may produce massive fusion of host cells that are converted into a multinucleated syncytium within minutes. This 'fusion from without' phenomenon does not require replication since it can be induced with a virus stock whose genomes have been inactivated by radiation. In fact, the fusion activity of certain viruses is so striking that it is used by cytogeneticists as a method for production of heterokaryons, involving the fusion of two different cell types to make a cell that contains two different nuclei. Sendai virus, a paramyxovirus, has frequently been used for this purpose. Figure 4.2 shows a typical virus-induced syncytium.

Many viruses cause abortive infections in certain cell types that fail to provide the required cellular proteins necessary to complete transcription, translation and assembly of new infectious virions. However, such abortive infections may lead to cell death via some of the pathways described later in this chapter. Again, productive virus replication and cellular alterations are not necessarily linked.

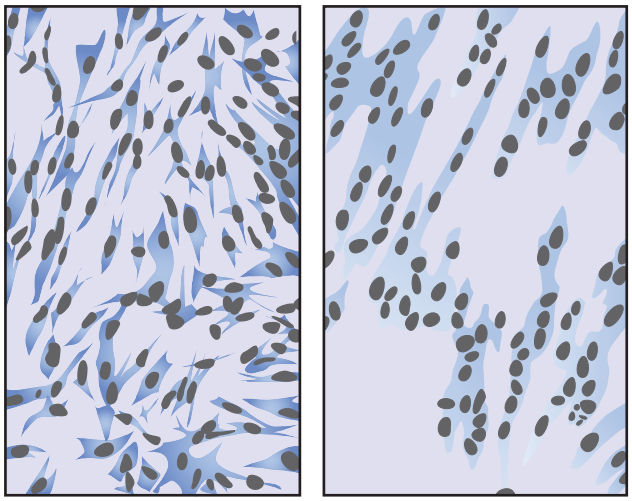

FIGURE 4.2 Syncytia produced by an enveloped RNA virus. Both panels show cell cultures exposed to La Crosse virus, a bunyavirus. The culture on the right was then treated with a low pH buffer for a few minutes to initiate the fusion process, while the control culture on the left was exposed to only a neutral buffer (fusion did not occur). Acid-dependent fusion is characteristic of many RNA viruses that only initiate fusion after they have undergone endocytosis and the pH has dropped in the endocytic vacuole. After Gonzalez-Scarano F, Pobjecky N, Nathanson N. La Crosse bunyavirus can mediate pH-dependent fusion from without. *Virology* 1984, 132: 222–225.

Percent apoptosis	Genome segment										Virus strain
	L1	L2	L3	M1	M2	M3	M4	S1	S2	S3	
3	1	1	1	1	1	1	1	1	1	1	T1L
6	3	3	3	3	3	1	3	1	3	3	EB145
2	3	3	1	3	1	3	3	1	3	3	EB121
97	1	1	1	1	1	1	1	3	1	1	1.HA3
48	3	3	3	3	3	3	3	3	3	3	T3D

TABLE 4.1 Differences in the capacity of reovirus strains to induce apoptosis are determined by the viral attachment protein σ1 that is encoded by the genome segment S1. Mouse fibroblasts (L929 cells) were infected with type 1 Lang (T1L) and type 3 Dearing (T3D) strains of reovirus and reassortants between them and the degree of apoptosis was determined by the proportion of cells that showed characteristic chromatin changes (condensed or fragmented) when stained with acridine orange

After Tyler KL, Squier MKT, Rodgers SE *et al.* Differences in the capacity of reovirus strains to induce apoptosis are determined by the viral attachment protein σ1. *Journal of Virology* 1995, 69: 6972–6979, with permission.

CELLULAR RESPONSES TO VIRUSES

Responses to viral attachment

Fusion induced by binding of enveloped viruses to host cells has just been described. A number of other effects can also follow binding of a virus to its cellular receptor. Apoptosis, or programmed cell death, is described later in this chapter. Reovirus can initiate apoptosis just by binding to certain cell types and genetic studies show that the σ1 viral attachment protein of reovirus is responsible for this effect (Table 4.1). Likewise, the viral attachment protein of HIV-1 (the SU or surface envelope protein, gp120) can induce apoptosis by binding to its cognate receptor, the CD4 molecule. Experimental evidence indicates that this effect is due to crosslinking of individual CD4 molecules on the cell surface and the effect can be mimicked by anti-CD4 antibodies.

Many viral receptors are cell surface proteins whose function is to initiate transmembrane signaling, beginning a cascade of intracellular biochemical steps. In some instances, attachment of the viral surface protein will mimic the effects of the 'normal' ligand for the receptor and initiate the signaling cascade. For instance, the gp120 envelope protein of HIV-1 uses members of the chemokine receptor family, such as CCR5 and CXCR4, as a viral coreceptor. In some instances, binding of gp120 to CCR5 or CXCR4 will initiate transmembrane signaling events, as evidenced by activation of intracellular pathways or biological effects such as chemotaxis or apoptosis.

Viral modulation of host cell transcription

Viral infection can affect RNA transcription of host cells in different ways, depending upon the virus–cell combination. There may be a widespread downregulation of transcription, or a mixed up- and downregulation of different genes. These activities can lead to death of the infected cell, alterations in the cellular phenotype, or transformation and potential tumorigenesis.

Many viruses initiate a general reduction of host cell transcriptional activity that often begins soon after infection. Although long known and much studied, the molecular mechanisms of this phenomenon are not completely understood. A well documented example is infection with poliovirus, which rapidly induces a reduction in transcription by the three major RNA polymerases, Pol I, Pol II and Pol III, that catalyse the synthesis of ribosomal, messenger and transfer RNA, respectively. Poliovirus induces a reduction in the availability of transcription factors, as well as a decrease in the amount of polymerase associated with the DNA templates, leading to an acute drop in the rate of synthesis of mRNAs for many cellular proteins.

In some instances, viral infection decreases the transcription of a specific gene in a differential manner. One of the most intriguing examples is the ability of certain strains of lymphocytic choriomeningitis virus (LCMV) to cause growth retardation in suckling mice (Figure 4.3).

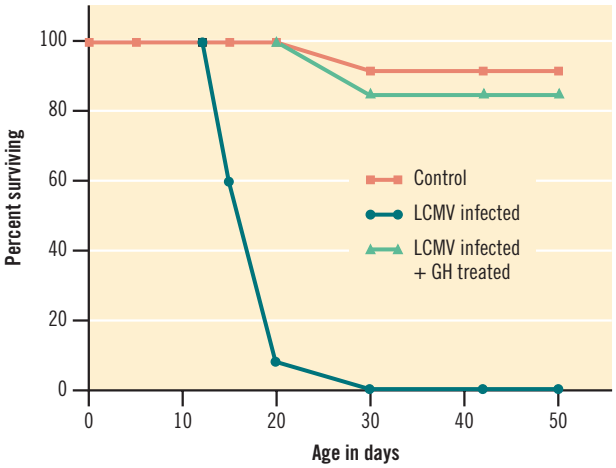

FIGURE 4.3 The effect of infection with lymphocytic choriomeningitis virus (LCMV) on the survival of infant mice is due to a reduction in the production of growth hormone (GH). GH has two major functions, to enhance normal growth and to regulate glucose metabolism. LCMV-infected mice exhibit retarded growth (not shown on this graph) and hypoglycemia that leads to death within one month of age. After Oldstone MBA, Rodriguez M, Daughaday WH, Lampert PW. Viral perturbation of endocrine function: disordered cell function leads to disturbed homeostasis and disease. *Nature* 1984, 307: 278–281, with permission.

DNA probe for	Function of protein	Relative amount of RNA		Ratio LCMV/ Uninfected
		Uninfected	LCMV	
Actin	Housekeeping	0.11	0.10	0.9
Collagen	Housekeeping	0.96	0.46	0.5
TSH	Hormone	1.72	0.69	0.4
GH	Hormone	3.80	0.24	0.08

TABLE 4.2 Transcription of specific mRNAs from the nuclei of pituitary cells isolated from normal and LCMV-infected mice reveals a differential reduction of the expression of the message for growth hormone. The table shows the relative amounts of hybridization of P^{32}-labelled nuclear runoff products from pituitary nuclei isolated from mice infected 15 days previously with LCMV compared with uninfected control animals
TSH: thyroid-stimulating hormone; GH: growth hormone. After Klavinskis LS, Oldstone MBA. Lymphocytic choriomeningitis virus selectively alters differentiated but not housekeeping functions: block in expression of growth hormone gene is at the level of transcriptional initiation. *Virology* 1989, 168: 232–235, with permission.

This effect was traced to a reduced production of growth hormone (GH) by the pituitary gland and it was shown that LCMV produced a non-lytic persistent infection of the GH-producing cells of the pituitary. LCMV infection was associated with a reduced level of GH mRNA and, surprisingly, this effect was messenger-specific because the mRNA levels for another hormone, prolactin, and for 'housekeeping' genes, such as actin, were reduced much less (Table 4.2). Genetic manipulation showed that the viral effect was associated with a domain in the GH promoter that bound a specific transactivator, although the precise molecular mechanism has yet to be determined.

Some viruses have a differential effect on the expression of selected host genes. Using a general method to detect changes in levels of mRNA, it was shown that human cytomegalovirus (CMV) infection was followed by changes in transcription of ~5% of cellular genes; approximately an equal number of genes exhibited enhanced or reduced transcription. These changes produced many different effects, most of which have yet to be studied. For instance, CMV infection drives host lymphocytes into cell division and some genes associated with G0 (cells in the vegetative state) are downregulated while other genes associated with the cell cycle, such as the cyclins, are upregulated.

Certain viruses increase transcription of cellular genes, often in a selective manner. For instance, HTLV-1 (human T cell lymphotropic virus type 1) can transform CD4 positive lymphocytes and this activity has been traced to the tax protein, a non-structural viral protein. Tax, by itself, can transform the cellular phenotype when transfected into T lymphocytes. Tax apparently acts indirectly, by binding to cellular transcription factors and increasing their ability to upregulate host cell transcription. It has a similar effect on the viral LTR (long terminal repeat, the viral promoter), which contains an element called CRE (cAMP responsive element) that binds a transactivator, CREB (CRE binding). When tax associates with CREB, it increases its ability to bind CRE and (in association with a co-activator, CPB–CRE-binding protein) enhances the transcription of viral genes.

Viral modification of host cell protein synthesis

As noted above, many viral infections are associated with the downregulation of host cell protein synthesis. This is accomplished through a wide variety of biochemical mechanisms, most of which interfere with the initiation of translation of mRNA. Yet, translation of viral messages is maintained, to insure the production of large amounts of structural proteins for assembly into nascent virions. How is this accomplished? Poliovirus, one of the best studied examples, illustrates the process.

Poliovirus and many other picornaviruses contain a viral protease that markedly reduces synthesis of host proteins. The protease cleaves a cellular protein essential for the binding of the ribosome to mRNA, a step required for the initiation of translation. Viral messages are translated by a mechanism different from host RNA messages, involving an internal ribosomal entry site (or IRES element) and this process does not require the cellular protein cleaved by the viral protease (Figure 4.4). This ingenious arrangement permits the virus to interfere with host protein synthesis while ensuring the continued synthesis of viral proteins.

Virus-induced alterations in cellular membranes

Cell fusion, described above, is probably the most striking effect of viruses upon cellular membranes. There are a number of other important effects that viruses can have upon the membranes of host cells.

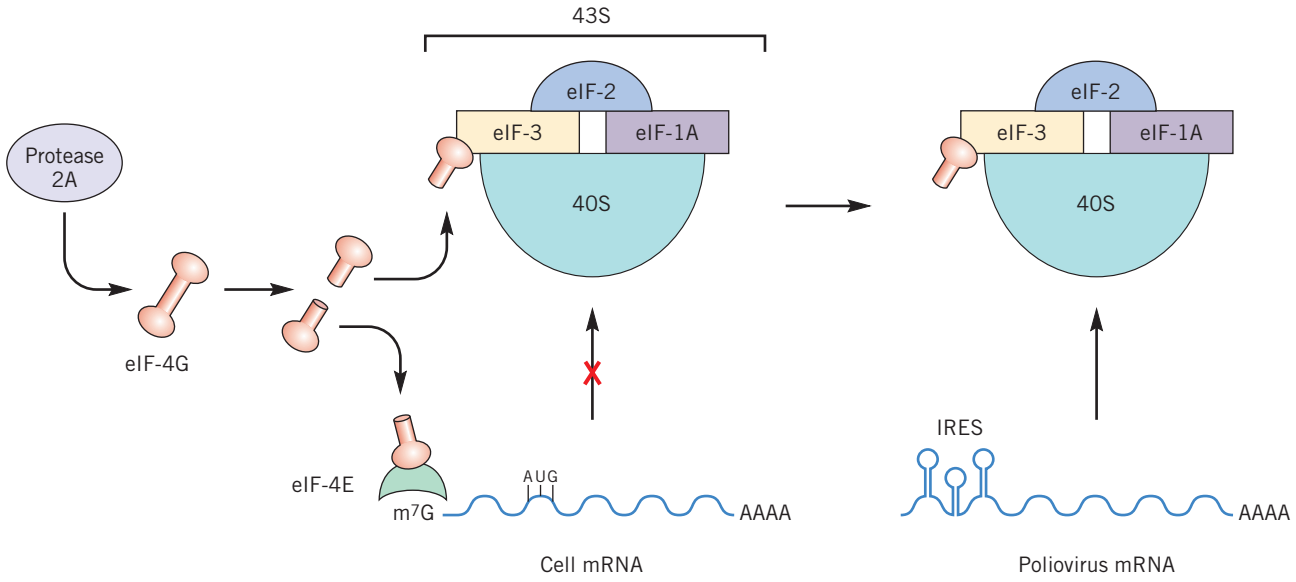

FIGURE 4.4 Picornaviruses inhibit host cell protein synthesis by the action of a viral protease, protein 2A, that cleaves a cellular protein, eIF4G (eukaryotic Initiation Factor 4G). Cellular mRNA molecules are modified (capped) at their 5′ end and are very poorly translated. Polioviral messages are not capped but contain an internal ribosomal entry site (IRES) that is recognized by the ribosome in the absence of eIF4G, leading to efficient translation of viral mRNA. eIF: eukaryotic Initiation Factor; IRES: internal ribosomal entry site; 40S, 43S: ribosomal complexes. After Figure 4.2 in Hardwick JM, Griffin DE. Viral effects on cellular function, Chapter 4 in Nathanson N *et al.* (eds), *Viral pathogenesis*, Lippincott Raven Publishers, Philadelphia, 1997 and Prevot D, Karlix J-L, Ohlmann T. Conducting the initiation of protein synthesis: the role of EIF4G. *Biology of the Cell* 2003, 95: 141–156, with permission.

Blocking or downregulation of the cellular receptor for virus

Animals or cells infected with avian sarcoma leukosis viruses (ASLV) are known to resist superinfection by other closely related strains of retroviruses and this phenomenon is used to classify these viruses into groups that exhibit reciprocal resistance. An infected resistant cell line sheds high titers of virus as well as many copies of its envelope protein, sufficient to saturate the specific viral receptor on the cell surface. This renders the cells resistant to superinfection by viruses that utilize the same receptor, while retroviruses that use different receptors can still infect such cells (Figure 4.5).

A related phenomenon has been described for HIV, a member of the lenti (slow) subgroup of retroviruses. Quantitation of the expression of CD4 – the primary viral receptor – on the surface of HIV-infected cells shows that CD4 expression is reduced by at least 10-fold in comparison with uninfected cells. Three viral gene products contribute to this effect; early in infection the *Nef* (negative factor) gene and late in infection the *env* (envelope) and the *vpu* genes downregulate surface expression of CD4. The three viral proteins probably act by quite different mechanisms (Figure 4.6). The envelope glycoprotein complexes with nascent CD4 in the endoplasmic reticulum and vpu increases the degradation of CD4 trapped in such complexes, while Nef induces endocytosis of CD4 that has already reached the plasma membrane.

Modulation of major histocompatibility complex (MHC) expression

Some viruses persist in vivo in spite of an immune response that includes virus-specific cytolytic T lymphocytes. As described in Chapter 6, virus-infected cells digest viral proteins and transfer oligopeptides to the endoplasmic reticulum where they complex with class I MHC (major histocompatibility complex) molecules. The MHC-peptide complexes migrate to the cell surface where they are recognized by the antigen-specific receptor on cytolytic T lymphocytes, which then may attack the virus-infected cells. Therefore, it is surprising that virus-infected cells can persist in vivo. In some instances, the viral infection down-modulates expression of class I proteins on the cell surface, thereby reducing the amount of viral peptides that can be displayed on the cell surface. Presumably, this enables infected cells to resist attack by the antiviral cellular immune response.

One interesting example of this phenomenon has emerged from investigation of the Nef protein of HIV-1. Nef is a small non-structural protein, the function of which is somewhat enigmatic. Mutant strains of HIV-1 and SIV (simian immunodeficiency virus, the natural 'HIV' of monkeys), engineered to delete the *Nef* gene, are able to replicate in cell culture with the same kinetics as cognate Nef-expressing viruses. However, when monkeys are infected with SIV strains containing a point mutation in Nef, they quickly are replaced with viruses with a functional *Nef* gene, implying that Nef has an important function in vivo. If a large mutation is made in *Nef*, such that repair is impossible, the Nef-deficient virus exhibits an attenuated phenotype with reduced viremia and delayed or absent development of immunodeficiency. The Nef protein has several functions, one of which is the ability to downregulate the expression of MHC class I molecules on the surface of infected cells. It is postulated that this effect protects the virus against immune surveillance, thereby increasing its ability to replicate and hence, its virulence.

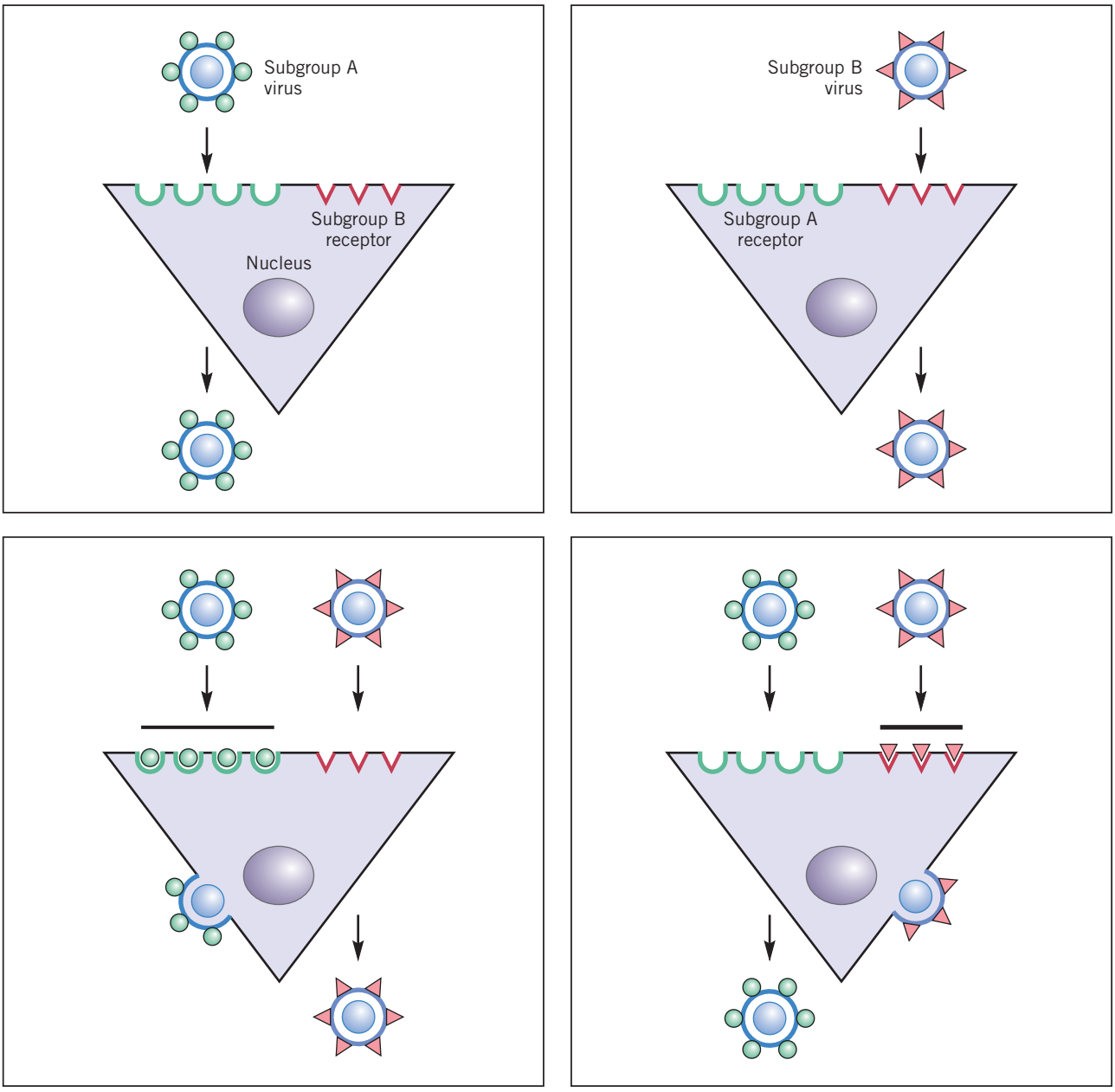

FIGURE 4.5 Blocking of infection among different retroviruses within the avian sarcoma/leukosis (ASLV) families. The four panels show the identical chicken cell bearing two sets of receptors, for subgroup A and subgroup B ASLVs, respectively. Top panels: uninfected cells are susceptible to either a subgroup A or subgroup B virus. Bottom left panel: cells previously infected with a subgroup A virus are resistant to superinfection with a second subgroup A virus, but retain susceptibility to a subgroup B virus. Bottom right panel: cells previously infected with a subgroup B virus are resistant to superinfection with a second subgroup B virus, but retain susceptibility to a subgroup A virus. After Coffin JM, Hughes SH, Varmus HE (eds), *Retroviruses*, Cold Spring Harbor Press, Cold Spring Harbor, 1997, with permission.

Conversely, the Nef-deficient virus is relatively avirulent, presumably because virus-infected cells are more susceptible to immune destruction.

Another important example of downregulation of class I MHC is the adenoviruses. Some adenovirus serotypes produce tumors in normal mice while others do not (Table 4.3). The ability to produce tumors is related to immune surveillance, because strains of adenovirus that do not produce tumors in normal mice will produce tumors in immunocompromised mice, such as mice that have been thymectomized shortly after birth. Furthermore, direct measurements show that low levels of MHC class I proteins are expressed on cells transformed by oncogenic adenovirus strains while high levels are expressed on cells transformed by non-oncogenic strains of virus. Together, these observations suggest that oncogenic strains of adenovirus downregulate MHC class I expression permitting adenovirus-transformed cells to escape immune surveillance.

How does adenovirus regulate the expression of MHC class I proteins, proteins which are a product of the cell not the virus? This trait is related to a single adenovirus protein, the E1A (early) protein, which has a dominant negative effect on MHC class I expression. In uninfected cells,

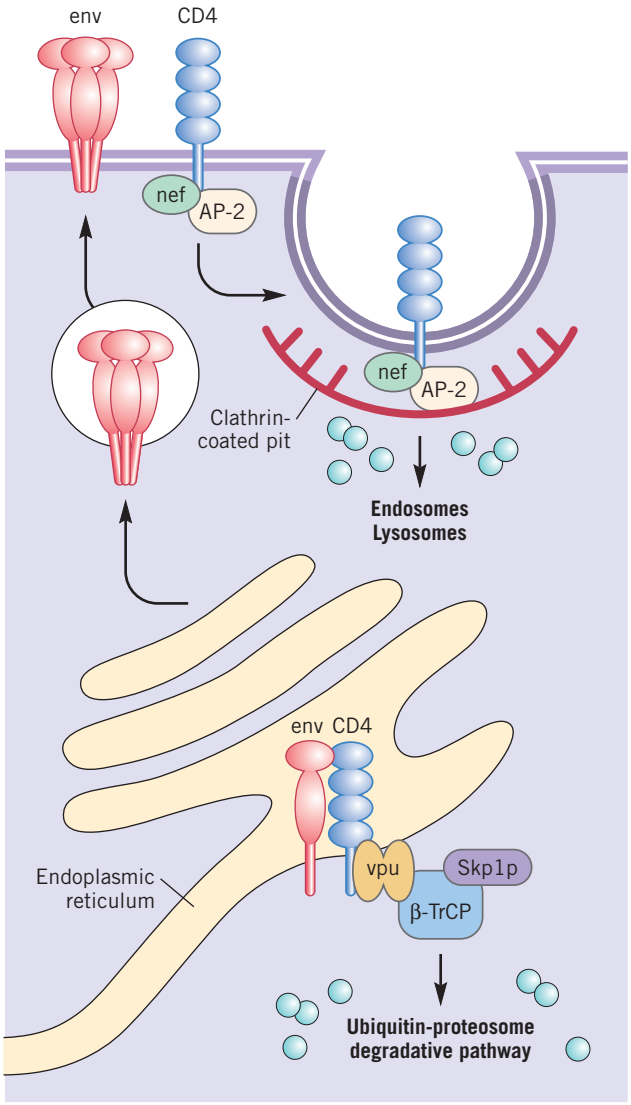

FIGURE 4.6 An example of viral proteins that downregulate the expression of a cell surface protein. CD4 is a cellular protein expressed on the surface of a subset of T lymphocytes that have a 'helper' function during immune induction; CD4 also acts as the primary receptor for HIV (see Chapter 3). These HIV proteins – Nef, env (gp160), vpu – combine to downregulate the expression of CD4 on the surface of HIV-infected lymphocytes. Nef binds to the cytoplasmic tail of CD4 and, through interactions with the AP-2 adaptor complex, directs CD4 to clathrin-coated pits, whence it is endocytosed and targeted to degradative lysosomes. Env binds to nascent CD4 in the endoplasmic reticulum and vpu recognizes the complex and, in conjunction with B-TrCP and Skp1p, directs it into the ubiquitin-proteosome degradative pathway. Skp1p: proteasome targeting factor; B-TrCP: protein that binds to Skp1p. After Emerman M, Malim MH. HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. *Science* 1998, 280: 1880–1884, with permission.

transcriptional activator proteins, such as NFκB, bind to DNA enhancer sequences just upstream from the MHC class I promoter and increase transcription of class I mRNA. The E1A protein of transforming adenoviruses interferes with this activity by modifying (possibly by dephosphorylating) NFκB, resulting in reduced expression of class I proteins on the surface of transformed cells. The E1A protein of non-tumorigenic adenoviruses lacks the ability to downregulate class I expression. As a result,

Adenovirus Serotype *Tumorigenicity*	Tumors in		MHC class I expression on tumors
	Normal mice	Immuno-compromised mice	
Adenovirus 5 *Non-tumorigenic*	No	Yes	High
Adenovirus 12 *Tumorigenic*	Yes	Yes	Low

TABLE 4.3 Tumorigenic activity of different adenovirus serotypes correlates with downregulation of MHC (major histocompatibility complex) class I expression on adenovirus-transformed cells
After Ricciardi, R. Adenovirus transformation and tumorigenicity, Chapter 23 in Prem Seth (ed.), *Adenoviruses: basic biology to gene therapy*, R G Landes Co, Austin Texas, 1999.

non-oncogenic adenovirus serotype 5 will transform cells, but these cells are destroyed by immune surveillance mechanisms (Chapter 6).

Viral proteins that serve as ion channels

Another unusual effect of viruses on cellular membranes is the alteration of membrane permeability by the introduction of viral proteins that serve as ion channels. The best studied example is the M2 protein of influenza virus type A, which is incorporated in the viral envelope. When influenza virus is endocytosed during the process of viral entry (see Figure 3.8), the drop in pH leads to a conformational change that converts the M2 protein into an ion channel, thereby conducting H+ ions into the interior of the virion. In turn, this internal drop in pH leads to a dissociation of the M1 (matrix) protein from the viral ribonucleoprotein (RNP) complex. This dissociation is essential to infection, because it frees the RNP complex for transport to the nucleus, a step required for the initiation of transcription of the viral genome.

Genomics analysis: an integrated approach

The introduction of microarray technology has facilitated a genomics analysis of the cellular response to viral infection (see Figure 1.4). It is now possible to scan large numbers of mRNAs to determine which genes show major deviations in transcription following infection. In turn, this provides insights regarding both the viral programming of cellular functions and the defensive response of the host cell. As mentioned above, analysis of the response of cultured leukocytes to human cytomegalovirus (HCMV) showed at least two categories of responding genes: first those involved in cell cycle regulation, oncogenesis and apoptosis; and second, those involved in regulation of the immune response. Also, exposure to inactivated HCMV caused the upregulation of a great many more genes than did infectious HCMV, suggesting that the virus encodes proteins that block the induction of some antiviral host responses.

VIRUS-INDUCED CELL DEATH: APOPTOSIS AND NECROSIS

In recent years, it has been recognized that there are two major pathways that lead to death of cells, necrosis and apoptosis. Necrosis is the long established term for cell death and can be induced by any severe insult to cells such as heating, cooling, unphysiological pH, other physical or chemical trauma, as well by the action of specific viral proteins. Necrotic cells can be identified by loss of membrane integrity with spilling of cellular contents and random degradation of DNA. The residual cellular debris is then engulfed by local phagocytes, producing a transient inflammatory scar.

Apoptosis, or programmed cell death, has been recognized in the last decade as a major alternative pathway to cellular destruction. Apoptosis is accomplished by a cascade of biochemical steps that produce several morphological stigmata, including blebbing of the plasma membrane (which remains intact in contrast to the membranes of necrotic cells) and condensation of chromatin around the periphery of the nuclear membrane. Terminally, the cell shrinks, condenses and breaks up into membrane-bound apoptotic bodies that contain cytoplasm, nucleoplasm or both. Importantly, the plasma membrane alters in such a way as to initiate phagocytosis and removal without the inflammation that characterizes necrosis. In addition, cellular DNA is enzymatically cut into nucleosome-length fragments of 180–200 basepairs, or multiples of this size, which produce a ladder when the extracted DNA is electrophoresed on agarose gels. The cytologic changes that are characteristic of apoptosis can be detected by histological tests developed for this purpose, such as the TUNEL assay.

Apoptosis, like many other cellular processes, is carried out by a sequence of intracellular events that can be defined at the cell surface or in the endoplasmic reticulum. The effector pathway usually is mediated by activation of a cascade of caspase proteases that produce the various stigmata of programmed cell death described above. Initiation of apoptosis is controlled by regulator and adapter proteins that are in a delicate balance. Healthy cells bind trophic factors to surface receptors which, in turn, sequester pro-apoptotic proteins (such as Bad); under these conditions anti-apoptotic proteins (such as Bcl-2) can inhibit the release of cytochrome c from mitochondrial membranes (that initiates the caspase cascade).

Originally, apoptosis was thought to be confined to programmed growth and development of organs and tissues. In addition to cell multiplication, cell migration and cellular differentiation, programs of normal development include the death of cells. Examples are formation of phalanges, development of the central nervous system and elimination of lymphoreticular cells with anti-self or other unwanted immune activities. More recently, it has been shown that viruses often kill cells by inducing apoptosis rather than via a necrotic pathway (Sidebar 4.2). Viral proteins can initiate apoptosis by a variety of molecular mechanisms, many of which are not understood in detail.

Initiation of necrosis by viral proteins

One example of a viral protein that initiates cellular necrosis is Ebola virus, a filovirus (negative-stranded enveloped RNA virus) that is maintained in a yet-to-be-identified animal reservoir in Africa. Rarely, Ebola virus crosses the species barrier to produce propagated human outbreaks that are associated with mortality as high as 50% of diagnosed cases. Ebola disease is an acute hemorrhagic shock syndrome and pathologically there is severe damage to the endothelial cells that constitute the walls of capillaries. The envelope glycoprotein (GP) of Ebola virus is the viral attachment protein that binds to the cellular receptor and determines its tropism for endothelial cells and monocytes. Quite separate from its attachment function, intracellular expression of the GP appears to be responsible for the cytotoxic effect of the virus. Ebola virus GP contains a serine- and threonine-rich mucin-like internal domain that appears to confer its toxic activity because constructs in which this domain has been deleted lack the toxic activity while maintaining other functions of the protein. It has yet to be determined how Ebola GP initiates necrosis.

Initiation of apoptosis by virus infection

It is now known that apoptosis can be induced at various steps in the virus life cycle, depending upon the specific virus. As mentioned above, reovirus can induce apoptosis via binding of the viral attachment protein, $\sigma 1$, to its cognate cellular receptor and HIV can induce apoptosis via binding to one of its coreceptors, CCR5 or CXCR4. Adenovirus initiates apoptosis early in its program of transcription, via protein E1A, and the tat protein of HIV, another early protein that acts as a transactivator of transcription of the viral genome, can also initiate apoptosis. The structural proteins of certain viruses, made later in

SIDEBAR 4.2

Apoptosis (programmed cell death) in viral infections

- Cells may die through two pathways, necrosis or apoptosis. These two routes to cell death can be differentiated by several cytological and biochemical criteria.
- Many viruses kill infected cells by inducing apoptosis rather than necrosis. Apoptosis can be initiated at different points in the infectious cycle, by specific viral proteins.
- Some viral proteins act directly to induce apoptosis while others act indirectly, by neutralizing the effect of a host cell protein that blocks apoptosis.
- In some instances, the difference between a virulent and an avirulent strain of virus is mediated by variation in the ability of a viral protein to initiate apoptosis or to overcome the effect of a host cell protein that prevents apoptosis.
- Some virus proteins can block the apoptosis pathway, leading to prolonged cell life and increasing the yield of progeny virions. Blocking of apoptosis is also a characteristic of some transforming viruses.

the replication cycle, may also induce apoptosis; examples are the envelope E2 protein of Sindbis virus, an alphavirus, and the VP3 protein of chicken anemia virus, a DNA virus.

In addition to direct induction of apoptosis, some viral proteins may induce apoptosis indirectly, by inactivating a host protein that blocks a constitutive cellular apoptosis pathway. A neurovirulent strain of Sindbis virus produces encephalitis and neuronal death in mice and the neurons are positive in the TUNEL assay indicating that neuronal death is mediated via the apoptosis pathway. By contrast, an avirulent strain of Sindbis virus fails to kill infected neurons. The *bcl-2* oncogene encodes a cellular protein that is known to block the apoptosis pathway in many normal cells and cell lines that express *bcl-2* resist the lethal effects of Sindbis virus while those that do not express *bcl-2* are often killed. A genetic recombinant strain of neurovirulent Sindbis virus, which expresses the *bcl-2* gene (in addition to viral genes), exhibits the phenotype of avirulent Sindbis virus when used to infect mice. These correlations suggest that the ability of virulent Sindbis virus to kill neurons may be due to its ability to overcome the anti-apoptotic effect of the host cell genes such as *bcl-2*.

Poliovirus is another example of a virus that initiates apoptosis via an indirect mechanism. The poliovirus 2A protein is a protease that has several activities required for preferential translation of poliovirus uncapped mRNA relative to cellular capped mRNAs (Figure 4.4). In addition, the 2A protease induces cellular apoptosis, presumably by cleaving selected cellular proteins, although the exact mechanism is yet to be determined.

Anti-apoptotic actions of viral proteins

Although a number of viruses can induce apoptosis in infected cells, some viral proteins block the apoptosis pathway, thereby prolonging the production of virus by infected cells. Several herpes, pox and adenoviruses produce apoptosis-blocking proteins. The adenovirus example is interesting because one viral protein initiates apoptosis while another protein blocks the apoptosis pathway. As illustrated in Figure 4.7, E1A, one of the transforming proteins of adenovirus, drives resting cells into cell cycle; at the commencement of cellular S phase, the intracellular environment becomes permissive for the replication of this small DNA virus. E1A, by itself, induces the transformed phenotype, manifested by the appearance of foci of continually dividing cells. However, these foci wither and die because E1A also induces apoptosis. However, E1B, another adenovirus protein, is capable of blocking the apoptosis pathway and cells co-transfected with both E1A and E1B are stably transformed.

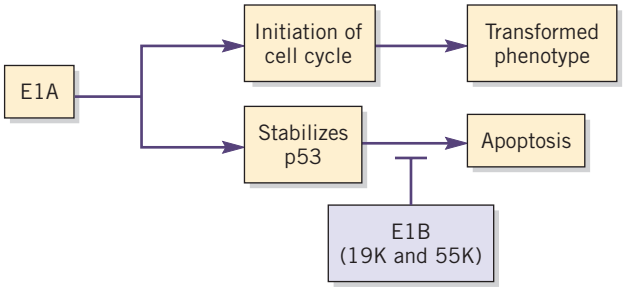

FIGURE 4.7 Some viruses produce proteins that both induce apoptosis and block apoptosis. The adenovirus E1A gene drives resting cells into cell cycle and thence into a transformed phenotype. The same gene product also stabilizes p53, an anti-oncoprotein, and p53 initiates apoptosis. Thus cells transformed with E1A alone undergo transient transformation but the transformed foci quickly die. Adenovirus E1B encodes a p19 and a p55 protein and both of these proteins block the apoptosis pathway. Cells co-transformed with both E1A and E1B genes undergo stable transformation.

HOW VIRUSES USE HOST CELLS

Viruses and the cell cycle

Many viruses replicate preferentially in cells that are in a specific phase of the cell cycle. The smallest viruses encode a very few proteins and are dependent upon the cell to provide a large number of the proteins required for replication of the viral genome and for the transcription and translation of viral messages. Often these required proteins are expressed only at a specific point in the cell cycle, limiting virus replication to cells at the required phase of the cycle. In some instances, the virus can control cycling of the cells that it has infected, in order to maximize its ability to replicate. One frequent strategy exhibited by a number of DNA viruses, whose genomes replicate solely or preferentially in cells in the S phase (DNA replicating phase) of the cycle (e.g. parvoviruses, adenoviruses, herpesviruses, papillomaviruses), is to induce infected cells to begin cycling, favoring replication of the viral genome.

One example is HHV8 (human herpesvirus 8), a virus associated with Kaposi's sarcoma, an unusual vascular tumor that occurs most frequently in patients with AIDS. HHV8 has a number of genes that are 'pirated' cellular genes, which are used by the virus to control the cell cycle. HHV8 expresses a GPCR (G-protein coupled receptor) that is active in the absence of any ligand and can transform cultured fibroblasts by activating one or several intracellular signaling pathways. Also, the viral GPCR initiates the synthesis and secretion of VEGF (vascular endothelial growth factor), a powerful angiogenic agent that is expressed by the spindle cells that characterize Kaposi's sarcomas. Further, several HHV8 genes lead to upregulation of the proteins that initiate cellular entry from G1 into S phase, leading to cell division (Figure 4.8). Additional examples of viruses that express modified forms of cellular proteins with transforming activity are discussed in Chapters 11 and 12.

Other viruses require (or prefer) cells that are at another point in the cell cycle. HIV preferentially infects CD4-positive T lymphocytes that are dividing, but it arrests the cell cycle in G2, probably because the cellular environment is then optimal for transcription from the viral LTR. This effect is mediated by the Vpr protein of HIV, a small regulatory protein expressed early after infection and has been attributed to inactivation of the cyclinB1/Cdc2 kinase complex that moves the cell cycle from G2 into mitosis.

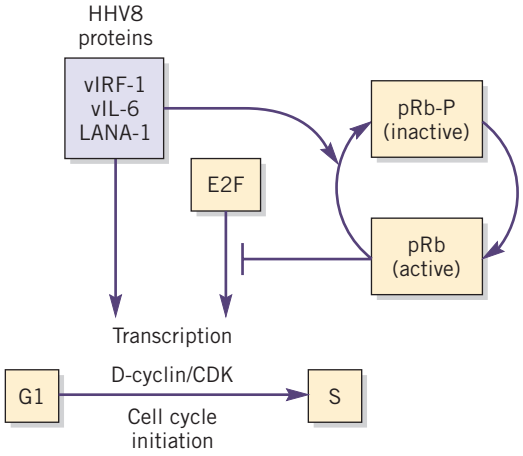

FIGURE 4.8 Viral proteins can initiate the cell cycle and stimulate proliferation of infected cells. In this example, several proteins encoded by HHV8 (human herpesvirus 8) initiate the cell cycle by direct stimulation of the transcription of cyclins and cyclin-dependent kinases (CDK) and by activation of proteins, such as retinoblastoma protein (pRb), that provide a brake on the cell cycle. E2F: family of related transcription factors; vIRF-1: viral interferon regulatory factor 1; vIL-6: viral interleukin 6; LANA-1: latency-associated nuclear antigen-1. After Direkze S, Laman H. Regulation of growth signalling and cell cycle by Kaposi's sarcoma-associated herpesvirus. *International Journal of Experimental Pathology* 2004, 85: 305–319, with permission.

Utilizing host chromosomes: Epstein-Barr virus (EBV)

EBV is a γ-herpesvirus that is maintained as a latent infection of B lymphocytes. The viral genome replicates once every cell cycle and the newly synthesized genomes segregate into daughter cells, but the virus does not encode enzymes needed for DNA replication. Instead, an EBV protein, the EBNA-1 protein (Epstein-Barr nuclear antigen) tethers the viral origin of replication to the mitotic spindles of replicating host cells. This insures both the replication of the viral genome in tandem with that of the host cell and the accurate segregation of viral genomes into daughter cells.

Viral interactions in proliferating cells: measles virus and human immunodeficiency virus (HIV)

It is well known that HIV replicates more robustly in proliferating than in resting CD4+ lymphocytes (see Chapter 14). An unusual example of this phenomenon is co-infection with HIV and measles virus. Measles virus infection of humans is characterized by transient profound immunosuppression but, paradoxically, when HIV-infected children are infected with measles there is a transient dramatic decrease in the level of plasma HIV. This phenomenon can be reproduced in cell cultures of blood lymphocytes that are first infected with HIV and later superinfected with measles virus. Measles virus infection induces a significant decrease in the proliferation of T lymphocytes accompanied by a significant reduction in HIV replication.

Viral exit from host cells

To perpetuate itself, a virus must be released from infected host cells and freed to spread further. For several reasons, release can pose tactical problems for viruses. For instance, they may be trapped within infected cells if the virus is not cytolytic, or they may rebind to receptors on the surface of the cell from which they are emerging, or they may be confined within a solid tissue. To circumvent these potential restrictions, viruses have evolved a large variety of strategies, a few of which are described below.

Adenovirus fiber protein and escape from its receptor

Adenovirus infects the epithelia of the respiratory and intestinal tracts. After assembly, the virus tracks to the basolateral surface of infected epithelial cells, where it is released. In order to find its way back to the mucosal surface, it must breach the tight junctions between epithelial cells. Adenovirus solves this problem by using its surface protein to break intercellular junctions (Figure 4.9).

Poxviruses, microtubules and actin

Poxviruses have evolved elaborate pathways to move from their intracellular site of assembly to the cell surface and beyond. In this case, the virus uses two distinct sets of cellular transport and motility machinery to move from a perinuclear site to the cell surface, emerge on the cell surface and then be 'shot' into a neighboring cell (Figure 4.10). First, intracellular enveloped virus (IEV) that has been assembled in the perinuclear region binds to kinesin, a protein that moves 'cargo' along microtubules. This transports the IEV to the plasma membrane, where it fuses to lose an envelope and is transformed into cell-associated enveloped virus (CEV) at the cell surface. By a complex series of events, actin is assembled below the plasma membrane and the actin polymer produces a finger-like extrusion that pushes the virus particle toward or into an adjacent cell. This process enhances cell-to-cell spread of virions and can also be used to evade antiviral antibodies in the extracellular milieu.

HIV and the ESCRT system

Budding of enveloped viruses is a complex process and the molecular details are yet to be completely revealed. HIV (and a number of other viruses) utilize cell machinery that is designed to deliver proteins to late endosomes. This machinery includes three protein complexes (ESCRT-1, -2, -3, endosomal sorting complex required for transport). The gag protein on the surface of the virus core binds members of the ESCRT complex, which are required to facilitate assembly and budding of the mature virion, although the individual steps are not well defined.

HOST DEFENSES AND VIRAL ESCAPE

The ability of animal hosts to mount antigen-specific immune responses to viral infection has been recognized since the inception of modern virology (see Chapters 1 and 6). The importance of innate immunity as a more primitive non-specific host response has only been recognized in the last 15 years (see Chapter 5). Even more recently, the dissection of detailed molecular pathways of

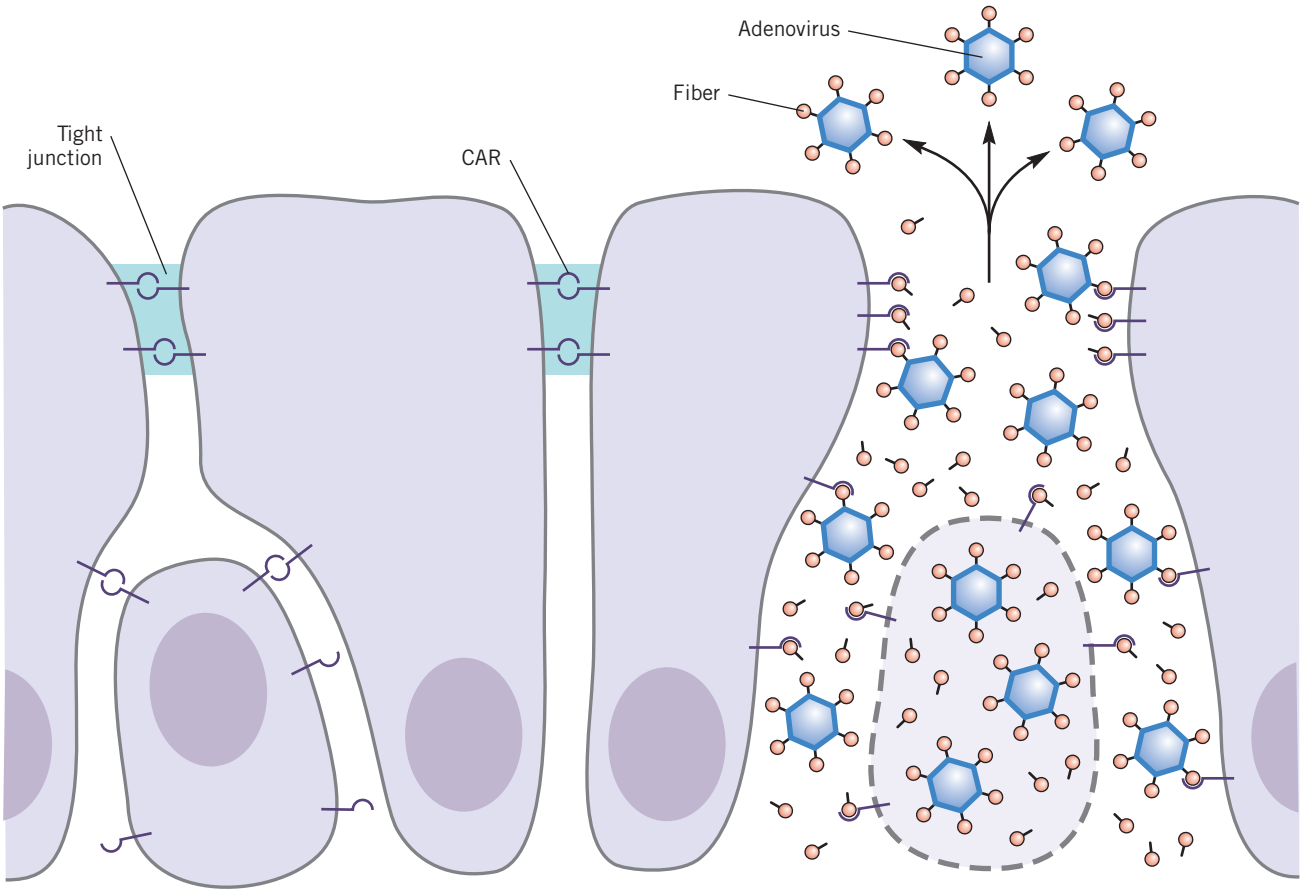

FIGURE 4.9 The fiber protein of adenovirus helps breach tight junctions between epithelial cells, permitting viral escape. Adenovirus enters epithelial cells by binding of its fiber protein to the coxsackie-adenovirus receptor (CAR) on the apical surface. The physiological role of CAR is to maintain integrity of tight junctions by establishing CAR-CAR bonds between epithelial cells. Adenovirus is released via the basolateral surface of cells where it is trapped by the tight junctions. However, the fiber protein binds to CAR, breaking the CAR-CAR bonds and permitting the virus to move towards the apical surface where it is free to spread to adjacent epithelial cells. After Walters RW, Freimuth P, Moninger TO *et al.* Adenovirus proteins disrupt CAR-mediated inter-cellular adhesion allowing virus to escape. *Cell* 2002, 110: 789–799, with permission.

viral infection at the cellular level have begun to reveal a third set of host defenses that attack individual steps in the replication cycle of individual viruses. Some examples are described below.

Non-immune host cell defenses and viral escape

Post-viral entry restriction: TRIM5α and HIV

Primates are naturally infected with a number of different lentiviruses, each of which is restricted to certain primate species. Thus, HIV-1 efficiently infects human cells that express the CD4 receptor and one of the coreceptors (CCR5 or CXCR4) (see Chapter 3). However, HIV-1 will not replicate in the cells of Old World monkeys. Investigating this restriction, it was found that HIV-1 will efficiently complete the entry phase of infection in restrictive non-human primate cells, as determined by internalization of viral RNA. However, the failure to generate any DNA transcripts identified a block prior to reverse transcription. Furthermore, studies with chimeric viruses indicated that the restriction involved the capsid proteins that form the outer layer of the viral core (see Chapter 14). Also, experiments with heterokaryons (cells formed by fusion of human and monkey cells) indicated that restriction was mediated by a dominant repressive factor, because such heterokaryons restricted HIV-1 infection. Using human cells transfected with rhesus monkey DNA, TRIM5α, a cytoplasmic protein of unknown function, was identified and shown to be the restrictive element. It has been postulated that TRIM5α binds a capsid protein and targets it for ubiquitination and proteasome-mediated degradation. The affinity of TRIM5α from different primate species for the capsids of different primate lentiviruses determines the patterns of species specificity.

Defense against foreign DNA: APOBEC3G and HIV Vif

The Vif protein is the product of one of the 'accessory' genes of HIV-1, a gene that is not found in many retroviruses, but is essential for HIV-1 to replicate in its major cellular target cells, CD4+T lymphocytes and macrophages. The elucidation of the function of Vif is a fascinating story and a monument to the ingenuity and perseverance of several investigators (Sidebar 4.3). Summarizing a complicated story, human cells contain a

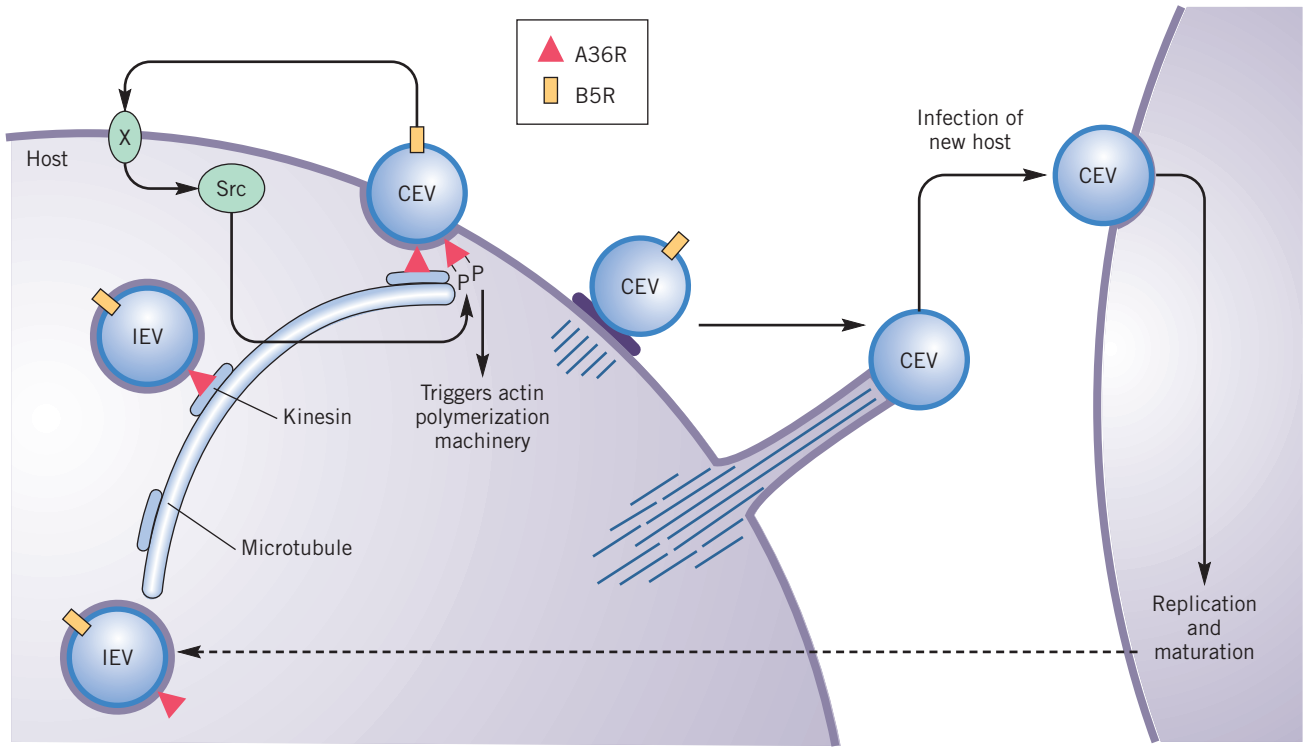

FIGURE 4.10 Vaccinia virus pathway between host cells. Vaccinia virus (IEV, intracellular enveloped virus) binds to kinesin, a motility protein that moves it along microtubules to the cell surface where it fuses with plasma membrane to become cell-associated enveloped virus (CEV). At this point, through a series of steps involving src (a cellular kinase), kinesin is released and the actin polymerization machinery is recruited to drive the virus on a finger of emergent cytoplasm that may embed it in an adjacent cell. A36R, B5R: vaccinia proteins; X postulated cellular protein; all of these proteins are involved in the intracellular transport of vaccinia virus. After Hall A. Src launches vaccinia. *Science* 2004, 306: 65–67 and Newsome TP, Scaplehorn N, Way M. Src mediates a switch from microtubule to actin-based motility of vaccinia virus. *Science* 2004, 306: 124–129, with permission.

SIDEBAR 4.3

The HIV accessory protein, Vif, is discovered to act by overcoming the antiviral action of a cellular protein, APOBEC3G

'After the human immunodeficiency virus (HIV) was first isolated in the early 1980s, the sequencing of its genome rapidly delineated nine genes, the products of which were soon assigned a variety of structure, enzymatic, or regulatory functions. All but one, *Vif* (virion infective factor, initially called *sor*), would largely elude the perspicacity of researchers for close to another 20 years. It did not take long to demonstrate the ~200-amino acid-long Vif protein, which accumulates in the cytoplasm of infected cells late in the viral life cycle, is important for virion infectivity, but more tedious efforts were required to reveal first that Vif-mutated virions can enter cells normally but yield markedly reduced levels of proviral DNA (the integrated form of the reverse-transcribed RNA genome) and second that this defective phenotype is entirely conditioned by the cell releasing the virus, not by its next target cell. ΔVif-permissive and ΔVif-restrictive cells were thus distinguished from each other, the latter, not surprisingly, was composed of the cells normally infected by HIV in vivo, namely, primary T lymphocytes and macrophages. An important breakthrough came with the demonstration that ΔVif restrictiveness is a dominant phenotype and, hence, reflects the intercellular antiviral activity specifically countered by Vif. Finally, through DNA subtraction, an approach requiring much patience, a member of the human cytidine deaminase family, APOGEC3G, was identified as this intracellular restriction factor. This discovery immediately set up intense efforts to elucidate the mechanism of APOBEC3G action against HIV and of its blockade by Vif. It ended up unveiling a far broader defense system than was initially suspected.'

From Turelli P, Trono D. Editing at the crossroad of innate and adaptive immunity. *Science* 2005, 307: 1061–1065, with permission.

protein, APOGEC3G, a cytidine deaminase that is incorporated into nascent HIV virions. When these virions enter a newly infected cell, reverse transcription of the viral RNA into DNA begins. APOBEC3G then acts upon the first (minus) DNA strand to deaminate cytidines, converting them into uridines, causing a guanosine to adenosine transition in the plus DNA strand. This results in hypermutation of the newly synthesized viral DNA and the resulting virions are not viable (Figure 4.11). The Vif protein counters APOBE3G by tethering it to proteins of the ubiquitin pathway, so that it is degraded and is not incorporated into nascent HIV-1 virions.

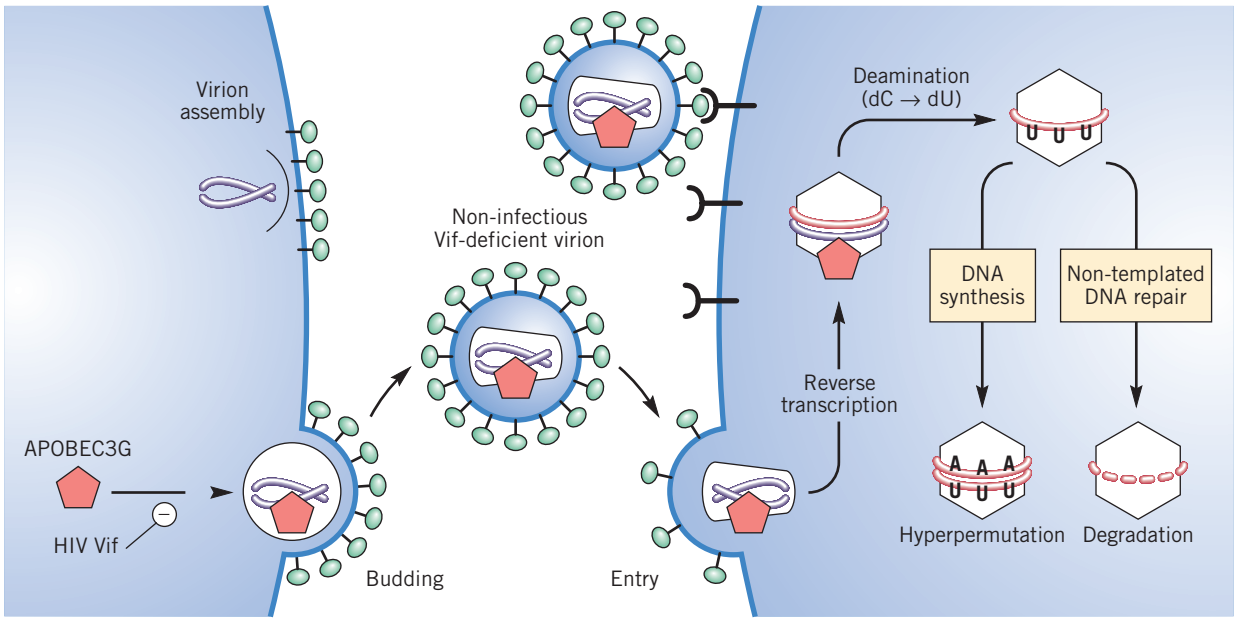

FIGURE 4.11 Cellular proteins with antiviral activity: APOBEC3G and HIV Vif. This cartoon shows the events when a susceptible cell is infected by an HIV-1 virus that lacks a functional *Vif* gene. APOBEC3G, a cellular cytodine deaminase, is incorporated into nascent virions. When these virions infect the next cell, the viral RNA is uncoated and reverse transcription is initiated. Cytodines in the first DNA strand are deaminated to uridine and the uridines are then paired with adenosine (rather than guanosine) in the second DNA strand. This perversion of the copying process leads either to guanosine-to-adenosine hypermutation or degradation of the nascent ds DNA. Wildtype HIV-1 has a functional Vif protein; Vif tethers APOBEC3G to cellular proteins that ubiquinate it and direct it to the proteosome where it is degraded (not shown in this diagram). APOBEC3G-free virions are then able to complete reverse transcription and evade this cellular defense mechanism. After Malim, personal communication, 2005 and Sheehy AM, Gaddis NC, Choi ND, Malim MH. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 2002, 418: 646–650, with permission.

RNA interference (RNAi) and viral response: HIV

It is well established that, under experimental conditions, siRNAs (small interfering RNAs) can be used to target viral sequences and that they have antiviral activity in cell cultures and animals (Chapter 16). Recently, it has been found that HIV-infected cells respond to infection by synthesizing virus-specific siRNAs. A computer-based analysis was used to identify potential double-stranded RNA sequences within the HIV-1 genome that might be targets for RNAi; one of these sequences was then shown to be present in HIV-infected (but not in control) cells and to have antiviral activity. RNAi is discussed in more detail in Chapters 5 and 16.

Host-mediated immune attack on virus-infected cells

As described above, many viruses replicate without causing death of the host cell. However, all infected cells express virus-encoded proteins, which can act as neoantigens that make the infected cell a potential target for antiviral immune responses directed against the viral proteins. Infected cells can present viral antigens in two ways. The surface proteins of enveloped viruses are expressed on the cell surface, often in patches that constitute sites for budding of mature virus. These viral envelope proteins can be recognized by antiviral antibodies. In addition, small peptides derived from viral proteins degraded by either an endogenous or an exogenous pathway, can be complexed with class I or class II MHC proteins on the cell surface where they are recognized as individual epitopes by T lymphocytes via their T cell receptors. (This process is described in more detail in Chapter 6.)

There is a multitude of specific immune responses that can attack virus-infected target cells (Figure 4.12). These include binding of antibody that triggers a complement–mediated attack on the target cell, lysis by NK cells that are 'armed' via their Fc receptors that bind to antiviral antibodies on the infected cell surface and lysis by cytolytic CD8+ effector T lymphocytes that recognize viral peptides bound to class I MHC molecules (see Chapter 6). In addition to the destruction of individual host cells, antiviral immune responses can cause a variety of inflammatory responses and pathological processes in vivo (see Chapter 7). Contrariwise, in some instances, the immune response can purge virus-infected cells without destroying them (see Chapter 6).

REPRISE

Virus infection can produce a wide array of effects on the host cell, from modulation of the expression of cellular genes to cell death. Viral replication does not necessarily cause cell death. However, cells can be killed by viral interactions – such as attachment and fusion – that may not result in viral replication. Cells can be affected by any stage in the viral life cycle, from attachment to release of new viral progeny and viruses may modulate every aspect of normal cellular function, including transcription, translation and cell division. Viruses often manipulate their host

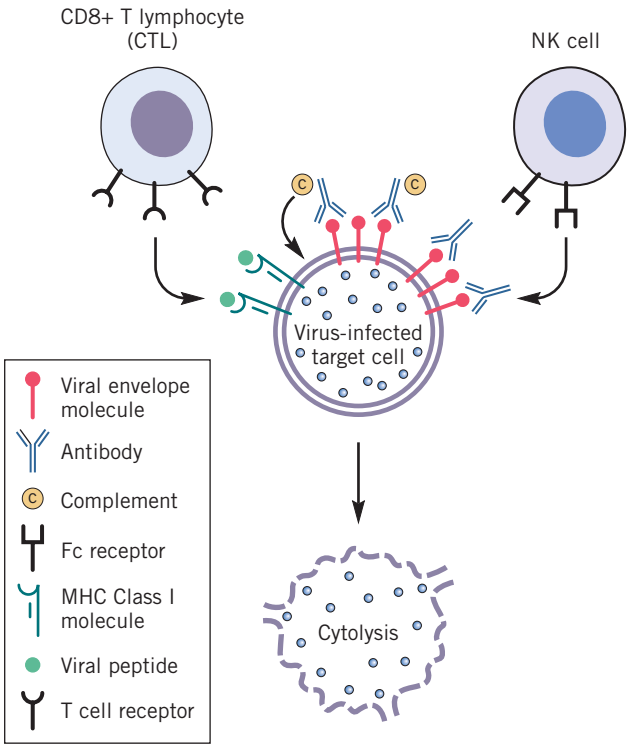

FIGURE 4.12 Cytolytic immune attack on virus-infected cells: selected mechanisms. Left: CD8+ T lymphocyte, via its T cell receptor (TCR) recognizes a viral peptide carried in the binding site of an MHC class I molecule, leading to activation of a lytic cascade of proteins secreted by the T lymphocyte. Middle: antibody binds to viral envelope proteins and initiates a complement cascade. Right: antibody binds to viral envelope protein and NK cells, via their Fc receptors, bind to antibody, triggering the release of toxic cytokines.

cells in order to facilitate their successful multiplication and escape. Virus-induced cell death can be the result of necrosis or apoptosis through a variety of different pathways. In an evolutionary response to virus attack, cells have developed a number of antiviral strategies and some viruses have evolved specific escape pathways. In summary, viruses utilize a plethora of mechanisms to enhance their ability to replicate in host cells and, as a result, the virus-cell interaction ranges from commensal to lethal.

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PART II

Host Responses to Viral Infection

Chapter 5

Innate Immunity

Neal Nathanson and Christine A. Biron

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REPRISE

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Following viral invasion, the infected host mounts a number of responses to infection. Many are a consequence of activation of adaptive immunity. These result in the production of antibodies and cells armed for defense with specificity for antigenic determinants expressed by the foreign pathogen. Infected hosts also possess a number of other defenses against an invading virus, which are often grouped under the umbrella 'innate immunity'. Although the components of the innate immune system were once thought to be non-specific, it is now clear that they have an elegant broad specificity very different from that of the adaptive immune system. In particular, innate immunity can be activated by chemical determinants not expressed on host cells and/or by products of cellular stress responses. Some of the innate effector molecules contributing to defense, such as interferons, are induced by infection, while others, such as complement and natural antibodies, exist prior to infection. In general, innate defenses come into play at the time of infection or shortly thereafter, filling an important gap in protection against viruses, until the appearance of the adaptive immune responses

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that characteristically require days to weeks for induction. Because of this delay, adaptive immunity is also known as acquired immunity. In addition, there are critical mechanistic connections between innate and acquired immunity, because some elements of innate responses set the stage for antigen-specific immune induction, by activation of professional antigen-presenting cells (APCs) and conditioning of adaptive immune cells.

REDISCOVERY OF INNATE IMMUNITY

Immunology has traditionally focused on the specificity of the acquired immune responses, in part because the elucidation of molecular mechanisms posed a daunting challenge that required many decades of research and the development of the tools and concepts of molecular biology. However, the idea of a more generic host defense was introduced over 100 years ago by Elie Metchnikoff, who recognized the power of 'phagocytes' to attack and destroy invading microbes. Furthermore, interferons – important components of innate immunity – were discovered in 1957, almost 50 years ago, by Alick Isaacs and immediately recognized as important for activation of significant host defense mechanisms with broad specificity. A critical advance was the identification, in 1973, by Steinman and Cohn of dendritic cells as a subtype of phagocytes that were specialized for the capture and presentation of antigen. Nonetheless, it was only in 1989 that Charles Janeway articulated a more holistic view of innate immunity, which synthesized past knowledge and added the concept of pathogen-associated molecular patterns (PAMPs) to define the essential chemical determinants broadly expressed on classes of pathogens, responsible for the induction of innate immune functions.

Interferons

In a classic series of experiments, Isaacs and Lindenmann reported that if the allantoic membrane from an embryonated chicken egg was exposed to inactivated influenza virus, an activity appeared in the supernatant fluids that, when applied to an uninfected membrane, could interfere with the ability of live influenza virus to infect the second membrane (Figure 5.1). Because the interfering activity was produced very rapidly, in the absence of immune cells and conferred resistance to many viruses, it could be considered the first identified molecular executor of innate resistance.

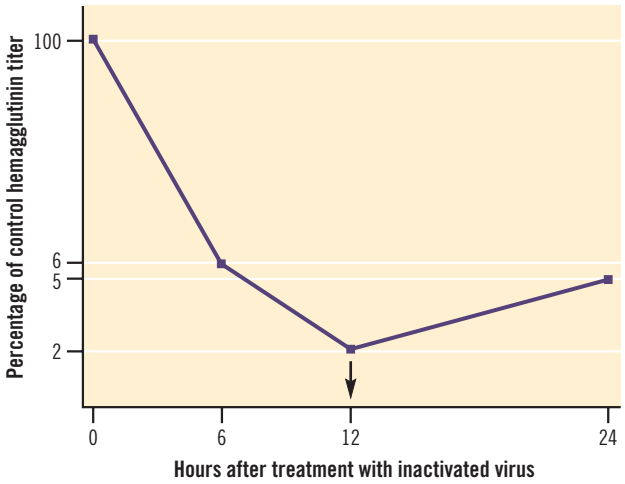

FIGURE 5.1 The discovery of interferon, an experiment showing the induction of interfering activity. Pieces of chorioallantoic membranes were exposed to heat-inactivated influenza virus and the membranes were removed after varying intervals. A fresh membrane was added to the medium, incubated for 24 hours, challenged with infective influenza virus and the medium was titrated 48 hours later for hemagglutinin activity. After Isaacs A, Lindenmann J. Virus interference. I. The interferon. II. Some properties of interferon. *Proceedings of the Royal Society of London B* 1957, 147: 258–273, with permission.

Pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs)

In his seminal 1989 commentary, Janeway noted the 'immunologist's dirty little secret', i.e. that most antigens would only elicit a brisk immune response when mixed with an adjuvant. The most successful adjuvants, such as Freund's complete adjuvant, contain microbial products (such as inactivated tubercle bacillus). Janeway proposed that such products included hypothetical PAMPs, repetitive molecular configurations that bound to pattern recognition receptors (PRRs) on 'innate' cells of the immune system (dendritic cells and other professional antigen-presenting cells) and activated them to participate in immune induction and defense.

Drosophila and the discovery of toll-like receptors (TLRs)

Drosophila, like all invertebrates, do not have a system for mounting acquired antigen-specific immune responses. Instead, they depend entirely on an innate system in which microbial challenge invokes the synthesis of a variety of antimicrobial peptides. Drosophila do encode a protein – dorsal – that is a member of the NFκB family. Because NFκB is a transcription factor that plays a critical role in immune induction in mammals, Jules Hoffman and his co-workers – in a jump of inspired intuition – decided to investigate whether genetic defects in the known regulatory cascade for dorsal would abrogate the ability of Drosophila to contend with their microbial pathogens. It was found that flies with mutations in the transmembrane receptor toll (at the proximal end of the dorsal cascade) were highly susceptible to fungal pathogens. (The name 'toll' [german slang for 'crazy'] was coined for a drosophila development mutant.) Following this lead,

Janeway and Medzhitov cloned a human homolog of toll (toll-like receptor, TLR) and showed that it was involved in the activation of NFκB in human cells. Bruce Beutler and associates then showed that mice with a genetic defect that made them unresponsive to bacterial lipopolysaccharide (LPS) – a notoriously pro-inflammatory microbial product – had a mutation in a TLR. This series of studies provided a mechanism – the TLRs – whereby a microbial product, broadly expressed on a class of pathogens but not on host cells, could activate immune cells and gave credence to the PAMP/PRR hypothesis.

Danger hypothesis

Polly Matzinger proposed another class of generic stimuli for the induction of innate immunity, which she christened the 'danger' hypothesis. The model suggested that stress or death of cells (in contrast to microbial products) can provide 'danger' signals to trigger the innate immune response. The hypothesis is consistent with the observation that most immunological adjuvants include a pro-inflammatory component, often associated with local tissue destruction. It appears likely that, as often happens in science, both the PAMP and the danger hypotheses are (at least in part) correct and should not be regarded as mutually exclusive competitors.

PRINCIPLES OF INNATE IMMUNITY

Cellular players: macrophages, dendritic cells and natural killer cells

Macrophages

Macrophages are phagocytic cells, derived from hematopoietic precursors in the bone marrow, released into the circulation as blood monocytes to enter organs to take up residence as tissue macrophages. In 1973, Steinman and Cohn identified a set of accessory cells required for immune induction, which they named dendritic cells (DCs), based on their morphology. Originally, it was assumed that DCs were derived from monocytes or macrophages, although it now appears that certain DCs may be derived from a lymphoid rather than a monocytoid cell lineage. Macrophages, dendritic cells and neutrophils (but not natural killer cells) express toll-like receptors (TLRs) as described below.

Dendritic cells

Dendritic cells play a central role in both innate and acquired immune responses. Currently (2005), several types of DCs are recognized, but information and terminology are still evolving (Figure 5.2, Table 5.1). All dendritic cells originate from hematopoietic precursor cells (HPCs) in the bone marrow.

Plasmacytoid dendritic cells (pDCs) appear to develop from a lymphoid precursor but their specific origins are still controversial. They circulate as plasma-like cells and then migrate into lymphoid tissue. pDCs differentiate under the influence of Flt3 ligand, a cytokine, and are identified by their CD11c-/low phenotype. In their resting or immature form, pDCs are poor antigen presenters; on stimulation by exposure to certain viral stimuli or

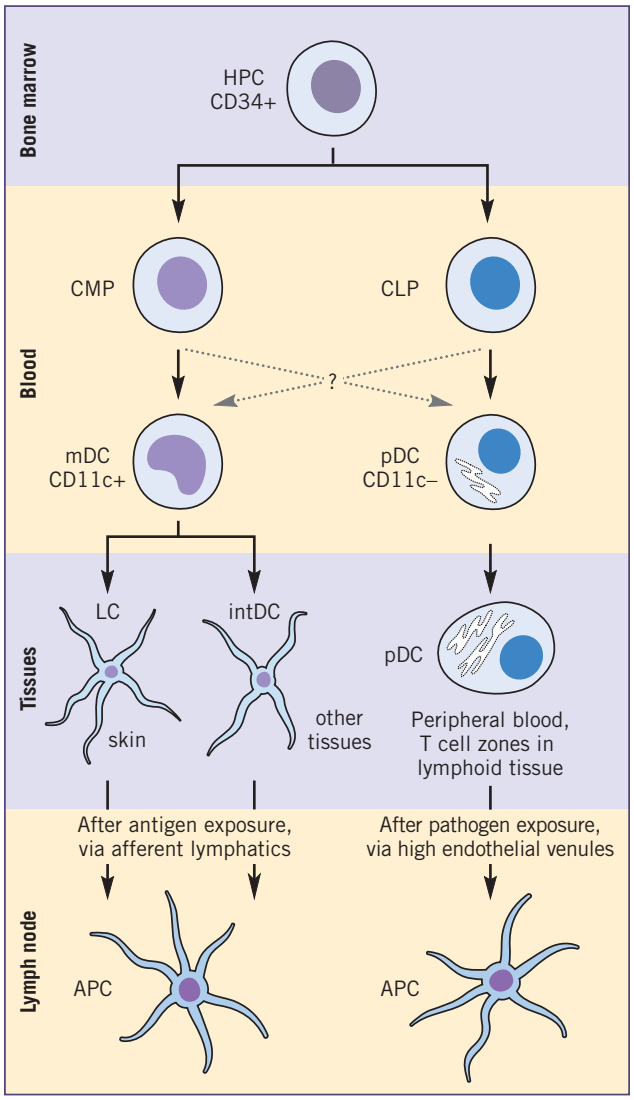

FIGURE 5.2 Human dendritic cell lineages. There may be some flexibility in the lineages shown in this figure. See text for description. HPC: hematopoietic precursor cell; CMP: common myeloid precursor; CLP: common lymphoid precursor; intDC: interstitial dendritic cell; pDC: plasmacytoid DC; mDC: myeloid DC; DC: dendritic cell; LC: Langerhans cell; APC: antigen presenting cell. After Banchereau J, Paczesny S, Blanco P *et al.* Dendritic cells, controllers of the immune system and a new promise for immunotherapy. *Annals of the New York Academy of Sciences* 2003, 987: 180–187; Colonna M, Trinchieri G, Liu Y-J. Plasmacytoid dendritic cells in immunity. *Nature Immunology* 2004, 5: 1219–1226; Liu Y-J. Dendritic cell subsets and linages, and their function in innate and adaptive immunity. *Cell* 2001, 259–262, with permission.

CD40 ligand, they secrete high titers of IFN-α/β (one of their hallmarks) and can act as professional APCs (antigen-presenting cells). However, it is not yet clear if or when they uniquely serve this function in the host.

By contrast, myeloid dendritic cells (mDCs) arise from circulating monocytes but there may be some flexibility in the lineages, and are identified by the CD11c+/bright phenotype. They migrate to tissues, including lymphoid tissues, where they take up a dendritic morphology. At least two subtypes are recognized, Langerhans cells (LCs) in the skin and interstitial DCs (intDCs) in other tissues. Blood monocytes, when cultured with GM-CSF and IL-4, differentiate into mDCs

Property	Dendritic cell type		
	Plasmacytoid	Myeloid	
		Langerhans	Interstitial
Drivers of differentiation from hematopoietic precursors	Flt3L, GM-CSF		GM-CSF, IL-4
Location	T cell zones of lymphoid tissues	Epidermis and dermis	Other tissues
Circulating surface markers	CD11c−	CD11c+	CD11c+
Activation required for efficient antigen presentation	Yes	Yes	Yes
Toll-like receptors	TLR 1, 6, 7, 9, 10	TLR 1, 2, 3, 5, 8, 10	TLR 1, 2, 3, 4, 5, 6, 8
Activation inducers	Viral RNA, viral DNA, CD40L	Antigen, inflammation, CD40L	Antigen, inflammation, CD40L

TABLE 5.1 Some properties of human dendritic cells. Different activation stimuli elicit somewhat different properties and 'cross differentiation' has been reported CD: cluster of differentiation; CSF: colony stimulating factor; Flt3L: fms-like tyrosine kinase 3 ligand; GM: granulocyte macrophage; IL; interleukin; TLR: toll-like receptor. After Banchereau J, Paczesny S, Blanco P *et al.* Dendritic cells, controllers of the immune system and a new promise for immunotherapy. *Annals of the New York Academy Sciences* 2003, 987: 180–187; Colonna M, Trinchieri G, Liu Y-J. Plasmacytoid dendritic cells in immunity. *Nature Immunology* 2004, 5: 1219–1226; Liu Y-J. Dendritic cell subsets and lineages, and their function in innate and adaptive immunity. *Cell* 2001, 106: 259–262; Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune response. *Nature Immunology* 2004, 5: 987–995.

and this preparation is used for most experiments with human DCs. The LCs appear to be further differentiated by the conditions in skin. Functionally, in their resting (or immature) state, all of these DCs are effective at binding antigens; when activated in peripheral tissues they migrate to draining lymphoid tissues and become effective professional APCs.

Natural killer (NK) cells

NK cells were originally identified in studies that described the ability of lymphocytes spontaneously to kill tumor cells in culture assays. This appeared to be an innate property since the cell donors had not been immunized with the tumor cells. Subsequent studies identified NK cells as a subpopulation of lymphocytes that could be distinguished from B and T lymphocytes, but had properties resembling T lymphocytes. Since that time NK cells have been recognized to play a significant role in innate immune response against pathogens, as described below.

NK cells are large lymphocytes that are defined by their cytoplasmic granules. These granules contain perforin – which can produce pores in plasma membranes and granzymes – proteins that can initiate apoptosis. When NK cells release their cytoplasmic granules, they can kill adjacent cells. These cells can also be induced to produce a variety of cytokines important for antimicrobial defense, including an immune interferon identified as IFN-γ and tumor necrosis factor (TNF). NK cells are identified by surface markers, the low affinity Fc receptor (FcγIIIR+), CD56+ and CD3−, that are used to isolate them for experimental purposes. NK cells do not express the T cell receptor (TCR) or immunoglobulins and therefore cannot produce an antigen-specific attack.

Instead, NK cells have activating receptors (not TLRs), currently being defined, that include integrins and other cell surface molecules. Activating receptors recognized to date are transmembrane proteins with short intracellular domains that do not directly initiate intracellular signaling, but are associated with signal-transducing molecules. When the NK receptor is occupied, the associated molecule initiates a signaling cascade that may lead to a release of their granules as well as the induction of transcription for the production of cytokines.

In addition, NK cells bear a large class of 'killer inhibitory receptors' (KIRs) which, when occupied, initiate intracellular signals that inhibit the activation pathways. Whether or not NK cells 'attack' is determined by the balance between the activating and inhibitory pathways. A large class of KIRs recognize MHC class I molecules and prevent NK cells from lysing most normal cells expressing these 'self' antigens. In contrast, cells that have reduced levels of MHC class I expression fail to activate KIRs and are targets for lysis (sometimes called the 'missing self' hypothesis). Certain tumor cells express reduced levels of MHC class I and thus are good targets for killing by NK cells, explaining their original identification as 'natural' killer cells. Many viruses will downregulate the expression of class I MHC (see Chapter 4) by a variety of mechanisms to avoid detection by cells of the adaptive immune system. NK cells appear to provide the host with a back-up for such a condition, because infected cells that have downregulated their class I MHC become more sensitive targets for attack by activated NK cells. However, certain viruses, particularly those with large genomes, encode genes that help to evade NK cell attack, either by mimicking MHC class I molecules (thereby activating KIRs) or by blocking NK cell activation receptors (see Chapter 9).

The mechanisms by which NK cells protect against viral infection is not thoroughly understood, but the

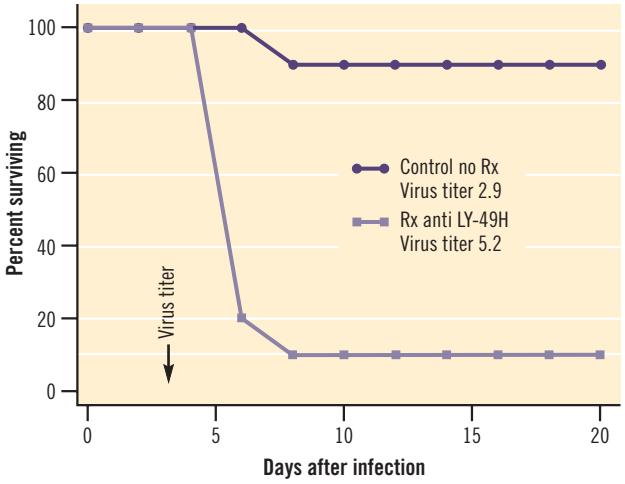

FIGURE 5.3 Depletion of NK cells renders mice more susceptible to an experimental infection. Mice were treated with a monoclonal antibody that was directed against an NK cell receptor, Ly-49H, and 2 days later were injected intraperitoneally with 10^5 PFU of murine cytomegalovirus. Mice were tested for virus titers ($\log_{10}$ PFU per spleen 3 days after infection) or were followed for survival. After Brown MG, Dokun AO, Heusel JW *et al.* Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science* 2001, 292: 934–937, with permission.

following tentative reconstruction can be ventured. NK cell activity is dramatically increased early in viral infection and this activation is mediated at least in part by type 1 interferons (α/β) that are rapidly induced by viral infection, followed by a complex set of activation events involving macrophages and cytokines. As just mentioned, activated NK cells will then attack virus-infected cells with reduced MHC class I expression. In addition, NK cell IFN-γ production can be induced during certain viral infections and this response can activate antiviral defense mechanisms and help promote inflammation at sites of viral infection. Thus, NK cells have multiple mechanisms in place to promote defense against viruses.

The importance of NK cells for defense against viral infections has been shown in both humans and mice. Humans with a genetic defect (Chediak-Higashi syndrome) that causes a selective defect in NK cells are prone to very severe herpesvirus infections. Furthermore, despite the presence of other arms of the immune system, a profound sensitivity to multiple herpesviruses has been identified in an individual with a complete absence of NK cells. Mice with a defect in NK cells or an NK cell receptor either by virtue of a genetic defect or by transient antibody-mediated depletion, exhibit increased susceptibility to murine cytomegalovirus (Figure 5.3).

In addition to their non-specific activity, NK cells can also mediate antibody-dependent cellular cytolysis (ADCC), an antigen-specific host defense that is described in Chapter 6.

Pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs)

Recently, several observations have revolutionized the field of innate immunity. Although these findings have focused on microbial rather than viral pathogens, information is accumulating on their function for innate immunity to viruses. Certain microbial cell wall constituents, such as lipopolysaccharides, lipoproteins and peptidoglycans, are found only in bacteria and not in eukaryotic cells. Components of these unique molecules have been called PAMPs, or pathogen-associated molecular patterns. As their name implies, PAMPs are patterns that are found in a large number of microorganisms. Since they recognize PAMPs, such receptors have been named pattern recognition receptors (PRR). PRRs are expressed on macrophages, DCs and neutrophils, all of which are important in host defense against microbial pathogens. One class of PRRs are the toll-like receptors (TLRs) described below.

Cells of the innate immune system respond in several different ways when their PRRs are ligated by a PAMP. In some instances, macrophages will phagocytose and digest foreign organisms, or they may initiate chemotaxis, bringing neutrophils to a site of inflammation. The TLRs usually initiate intracellular cascades that lead to secretion of cytokines (such as interferon, interleukins (IL-1, -6, -8, -12) and tumor necrosis factor (TNF-α)), which together orchestrate the inflammatory response against the foreign invader. An important response is the activation of DCs, followed by the binding of antigen, migration to draining lymph nodes and upregulation of co-stimulatory molecules (CD80, CD86), critical steps in the initiation of the acquired immune response (discussed below).

A defining characteristic of the innate immune response is its speed. Since PRRs are encoded in the germline on large classes of cells, they do not require genetic rearrangement or clonal expansion (in contrast to acquired immune responses). As a consequence, a large number of cells bearing a PRR can respond very rapidly (minutes to hours) to a foreign pathogen.

Toll-like receptors (TLRs)

TLRs are members of the toll family of molecules which have several common structural features, including an extracellular domain containing leucine-rich repeats and a cytoplasmic domain similar to that of IL-1 receptors. Some TLRs act in concert with other cell surface molecules to bind their ligands. TLRs signal through one of several intracellular cascades, which predominantly involve common components such as myeloid differentiation factor 88 (MyD88) or the toll-interleukin receptor (TIR).

Individual TLRs recognize specific microbial components that are not found in eukaryotic cells (Table 5.2). For instance, TLR4 recognizes lipopolysaccharide (LPS), a component of the cell walls of gram-negative bacteria; TLR2 recognizes peptidoglycan, a component of the cell walls of gram-positive bacteria; and TLR5 recognizes flagellin, a major component of bacterial flagella. TLR9 recognizes a DNA motif, unmethylated CpG (cytosine-phosphate-guanine). Since bacteria lack cytosine methylase, an enzyme found in eukaryotic cells, the unmethylated CpG motifs provide a microbial-specific ligand that binds to TLR9 and activates macrophages and DCs. It has long been known that CpG motifs have

an adjuvant activity, which is explained by their binding to TLR9. TLR9 can also respond to unmethylated DNA and viruses rich in DNA CG sequences may produce genomic material recognized by this receptor.

At the present writing, TLR3, 7 and 8 are the receptors that appear particularly relevant to RNA viral infections. TLR3 recognizes double-stranded (ds) RNA, an intermediate produced during the replication of many RNA viruses. TLR3 is mainly expressed on DCs and activation of DCs early in viral infection induces type I interferon (IFN), helping to explain the old observation that dsRNA is a potent IFN inducer. TLR 7 and 8 recognize single-stranded (ss) RNA, including viral mRNA and the genomes of ssRNA viruses.

In addition to the TLRs, there is a growing list of cytoplasmic receptors that activate signaling cascades in response to intracellular pathogens. The intermediaries of these cascades insert into downstream pathways to induce type 1 IFN. The most relevant for defense against viruses are the retinoic acid-inducible gene 1 (RIG-1) and the melanoma differentiation associated protein 5 (MDA5). Both of these proteins bind dsRNA to induce IFN. These receptors may be expressed in a wider range of cell types than the TLRs.

Interferon

The interferon (IFN) system is probably the most important of the broad host defenses against viral infection. Interferons are a group of proteins that are synthesized in response to viral infections and to other stimuli. Interferon induction occurs mainly at the level of transcription and the inducing molecules, directly or indirectly, act on promoters upstream from the interferon coding sequences. The interferon response is characteristically transient, even in the continued presence of the inducing stimulus.

Interferons do not produce their effects directly. IFNs are secreted and spread either locally or through the circulation to other cells where they induce the production of a characteristic set of many proteins that are responsible for the multiple effects of the IFN system (Figure 5.4). Of these effects, the most important are the inhibition of the replication of viruses and some other microorganisms, the inhibition of cell growth including that of certain tumor cells and the stimulation of the immune system by activating macrophages, lymphocytes and NK cells and upregulating the expression of class I MHC (major histocompatibility) molecules.

Interferons (IFNs) are classified as type 1 or type 2 (Table 5.3). Type 1 interferons (most importantly, IFNα and IFNβ) can be produced by all cell types if appropriately stimulated. They are typically induced by double-stranded RNA which is synthesized in the course of infection with many, but not all, viruses. TLR3 is an example of a receptor for dsRNA and, when engaged, it triggers an intracellular cascade that phosphorylates interferon regulatory factor 3 (IRF3), which translocates to the nucleus and activates transcription of IFN genes. Type 1 interferons are produced early in infection and therefore serve as a first line of defense prior to immune induction. Type 1 interferons can also be induced during infections with bacteria and parasites and by a variety of stimuli. In fact, the large family of type 1 IFNs, including one IFN-β and up to 14 IFN-α genes, may have evolved, at least in part, because of the diversity in their promoters. This provides complex regulation of gene expression

TLR	Cellular localization	Viral ligands	Bacterial ligands
1	Plasma membrane		Lipoproteins, peptidoglycans
2	Plasma membrane	Envelope proteins	Lipoproteins, peptidoglycans
3	Endosomes	ds RNA	
4	Plasma membrane	Envelope proteins	Lipopolysaccharide
5	Plasma membrane		Flagellin
6	Plasma membrane		Lipoproteins, peptidoglycans
7	Endosomes	ssRNA	
8	Endosomes	ssRNA	
9	Endosomes	Unmethylated CpG	Unmethylated CpG
10	Plasma membrane	Unknown (2005)	Unknown (2005)
11	Plasma membrane		Uropathogenic bacteria

TABLE 5.2 Toll-like receptors (TLRs) and their ligands. There are some differences between human and mouse TLRs (humans apparently do not express a functional TLR11; ssRNA may be recognized by mouse TLR7 and human TLR8) After O'Neill LAJ. After the Toll rush. *Science* 2004, 303: 1481–1482; Akira S, Takeda K. Toll-like receptor signaling. *Nature Reviews Immunology* 2004, 4: 499–511; Liew FY, Xu D, Brint EK, O'Neill LAJ. Negative regulation of toll-like receptor-mediated immune responses. *Nature Reviews Immunology* 2005, 5: 446–458; Pasare C, Medzhitov R. Toll-like receptors and acquired immunity. *Seminars in Immunology* 2004, 16: 23–26.

and the flexibility to use a number of different pathways for induction.

Type 2 interferon (IFNγ) or 'immune' interferon, can be part of either the innate and adaptive immune responses because it is a product of both the innate NK cells and the adaptive T lymphocytes if they are appropriately activated. Production is greatly enhanced by the presence of an innate cytokine, IL-12, but stimulation through other cell surface receptors can also induce IFNγ. Moreover, a number of cytokines, such as IL-2, and other intercellular mediators, such as estrogens, may activate IFNγ-producing cells.

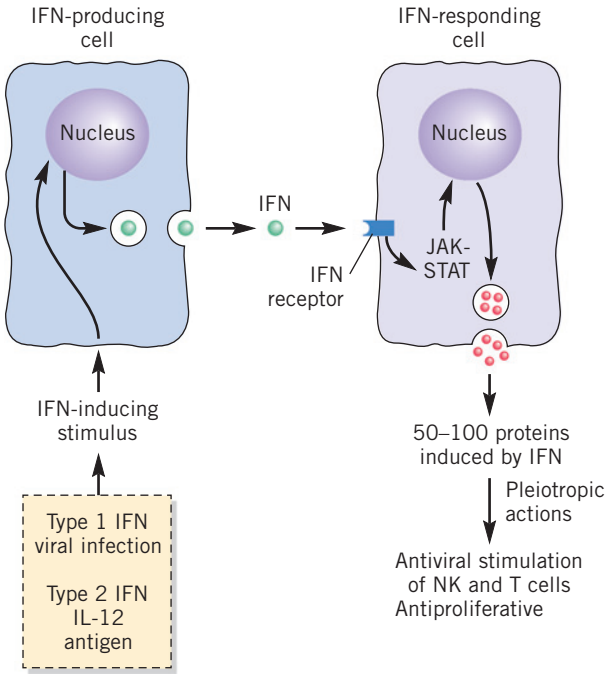

FIGURE 5.4 An overview of the interferon system. A number of different stimuli can induce cells to synthesize and secrete interferons; the stimulus for type 1 (IFNα/β) interferons is usually virus infection or double-stranded RNA, while the stimulus for type 2 interferon (IFNγ) is antigen stimulation through the T cell receptor and/or exposure of NK or activated cells to IL-12. Many cells can produce type 1 interferons, while type 2 interferons are produced mainly by T lymphocytes and NK cells. The secreted interferons bind to specific receptors, present on most cells, and induce the production of a characteristic range of proteins that mediate the pleiotropic actions of the interferons. See text.

Interferon receptors are found on most cell types and are distinct for type 1 and type 2 interferons. These interferon receptors are heterodimers and include an extracellular ligand domain, a transmembrane domain and an intracellular signalling domain. Characteristically, the receptors are specific for the interferon molecules produced by the same species, explaining the early observation that interferon produced by one species will not act on cells of another species. When interferons bind to their cognate receptors, an intracellular signaling cascade is initiated which involves the JAK-STAT pathway (Figure 5.5). Briefly, the intracellular signaling domain activates Janus tyrosine kinases (JAK) to phosphorylate signal transducer and activator of transcriptions (STAT). The phosphorylated STAT complex moves to the nucleus where it enhances the transcription of selected genes. Interferon induces the transcription of gene products within minutes of binding to the IFN receptor.

Viruses vary in their ability to induce interferons and in their sensitivity to interferon. Interferon induces more than 100 different proteins and a number of these proteins interfere with virus replication in different ways, many of which have not been elucidated. As might be predicted from the large number of induced proteins, different viruses are inhibited by different mechanisms. Inhibition can occur at the level of genome replication, transcription, mRNA stability or translation. One example is the Mx protein, a cellular protein induced by interferon that is known to be highly specific in its ability to depress the replication of influenza viruses.

Interferons may also have indirect effects upon virus infection through many immunoregulatory functions. Examples include activation of NK cell killing, induction of the cytokine IL-15 to enhance NK and T cell expansion, inhibition of non-specific T cell proliferation, enhancement of long-term T lymphocyte survival, enhancement of T cell IFNγ production and upregulation of class I MHC to render virus-infected cells more sensitive to immune surveillance by antigen-specific T lymphocytes. Some of these complementary or

Type	Cell source	Stimulus	Class	No. of subtypes	Chromosome
Type 1	All cells	Virus infection, dsRNA	α	12	9
			β	1	9
			ε	1	9
			κ	1	9
			ω	1	9
Type 2	T lymphocytes NK cells	IL-12 antigen	γ	1	12

TABLE 5.3 The human interferons and some of their properties. Type 1 interferons are structurally unrelated to type 2 interferon. Type 1 interferons are typically secreted in response to viral infection, triggered by intracellular appearance of double-stranded RNA (dsRNA). Type 2 interferon is typically secreted by T lymphocytes and NK cells following stimulation by IL-12 which, in turn, is secreted by macrophages and dendritic cells in response to antigen presentation and activation. This table does not include the IFN-like interleukins
After Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. *Immunological Reviews* 2004, 202: 8–33.

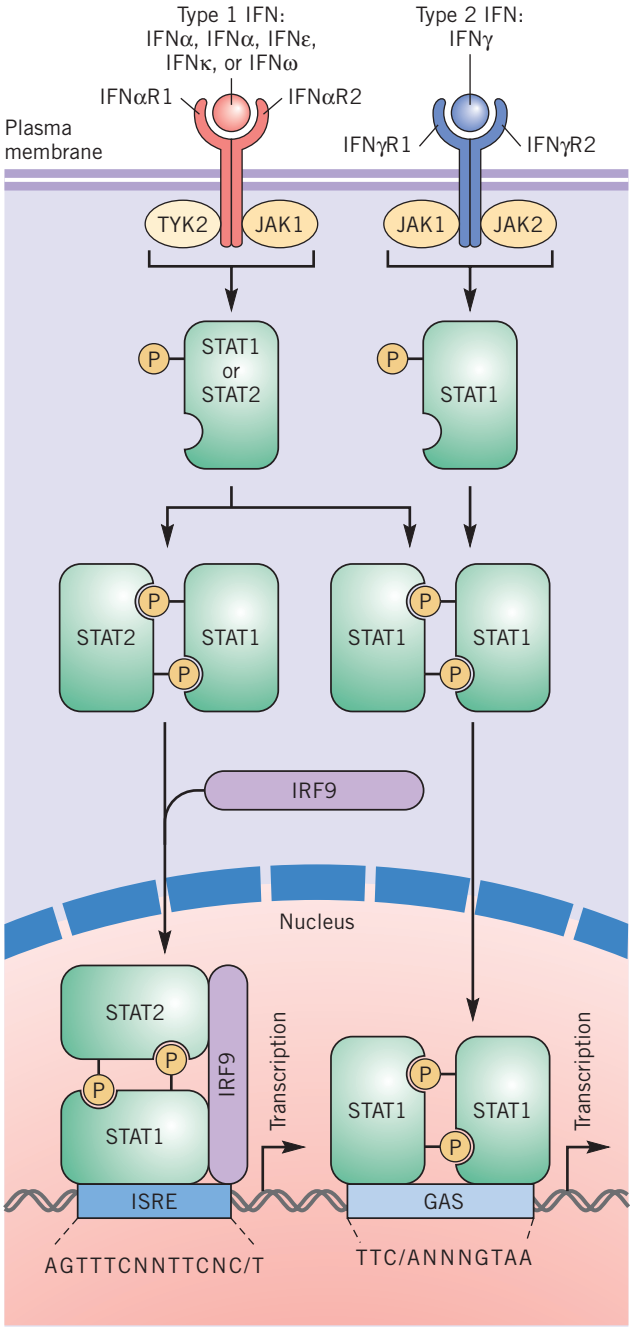

FIGURE 5.5 Intracellular pathways activated by IFNs. This diagram shows only the 'classical' JAK-STAT pathway. Type 1 and type 2 IFN each bind to their specific receptors and initiate phosphorylation of STAT1 or STAT2; activated STAT complexes migrate to the nucleus where they bind to specific DNA sequences and transactivate downstream genes leading to the production of proteins that mediate the pleiotropic responses to IFNs. IFNαR1, 2: type 1 IFNα receptors; IFNγ R1, 2: type 2 IFNγ receptors; JAK: Janus-activated kinase; TYK2: tyrosine kinase; STAT: signal transducer and activator; IRF9: IFN response factor 9: ISRE: IFN-stimulated response element; GAS: IFN gamma-activated site. After Platanias L. Mechanisms of type-I and type-II-interferon-mediated signaling. *Nature Reviews Immunology* 2005, 5: 375–386, with permission.

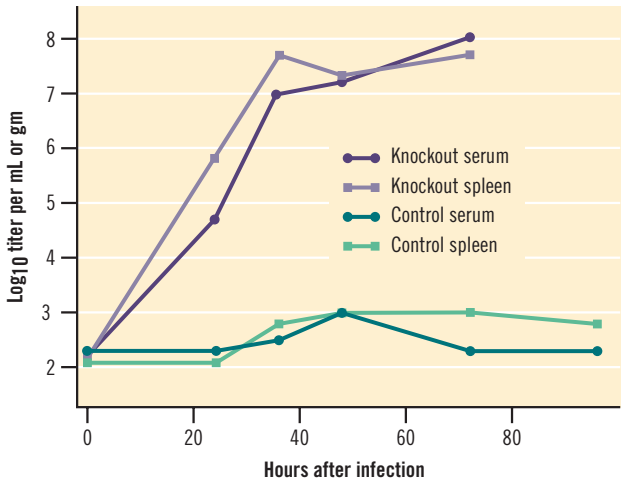

FIGURE 5.6 Enhanced susceptibility to Sindbis virus of mice lacking the receptor for type 1 IFN. Adult control mice (strain 129 Sv/Ev) and congenic knockout mice (strain 129 Ev/Sv INFα/βR$^{-/-}$) were infected with 100 plaque-forming units by the subcutaneous route and followed for virus titer and illness. Sindbis virus replicated mainly in macrophages and dendritic cells in many tissues and susceptible mice died very rapidly, perhaps from an overwhelming 'cytokine storm'. After Ryman KD, Klimstra WB, Nguyen KB, Biron CA, Johnston RE. Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism. *Journal of Virology* 2000, 74: 3366–3378, with permission.

paradoxical effects may be mediated by activation of different intracellular signaling pathways.

Interferon is particularly important for a host defense against primary virus infection, while specific immune responses, such as antibody, play a major role in early defenses against re-infection. The role of interferon can be demonstrated in animal models in which the interferon response is reduced by treatment with anti-IFN antibodies, or in mice whose interferon genes (or interferon receptor genes) have been 'knocked out'. In both instances, such animals, compared to controls, exhibit a reduced ability to contain virus infections and often show an increased incidence of illness or death (Figure 5.6). When the type 1 (IFNα/β) response is abrogated, there is a global increase in susceptibility to most viruses, while a knockout of the type 2 response (IFNγ) has a more modest effect, which is seen with some but not other viruses. These observations suggest that type 1 IFN plays a crucial role as a non-specific antiviral defense, whereas the type 2 IFN response may be only one element in a multicomponent immune effector system.

A number of viruses have evolved specific mechanisms to elude the effects of interferon and some of these are described in Chapter 9. The evolution and conservation of anti-interferon viral genes testifies to the importance of interferon as a host defense.

Defensins

Multicellular organisms produce 'natural' antimicrobial peptides that provide a defense against infection, particularly by bacteria and fungi. Among the known peptides are magainin (frogs), cecropin (silkworms) and the cathelicidins and defensins (best studied in primates and other mammals). Most of these molecules are amphipathic, composed of discrete hydrophobic and cationic (positively charged) domains. Their antibacterial action is explained by the interaction of the positively charged surface of the peptide with the negatively charged heads of

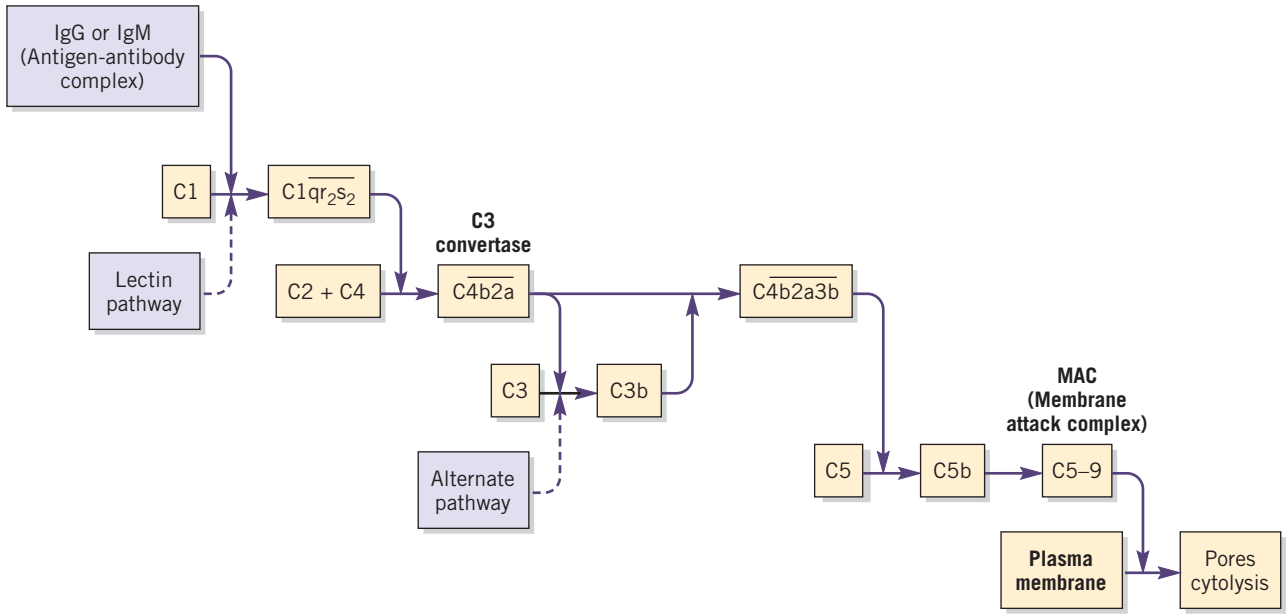

FIGURE 5.7 The complement cascade, an overview. The cascade can be initiated via the classical pathway, the alternative pathway or the lectin pathway, as indicated. The classical pathway is initiated by binding of antigen to antibody (either IgG or IgM) which produces a conformational change in the Fc portion of the antibody molecule enabling it to bind C1. The alternative pathway is triggered by specific carbohydrate residues on invading microorganisms that activate C3. The lectin pathway is triggered when a mannose-binding lectin (for instance, a θ-defensin) binds a cognate carbohydrate residue (on an invading microbe) followed by aggregation of enzymatic proteins that activate C2 and C4. At several steps in the cascade a complex of proteins is formed which has catalytic activity (indicated by the overbar). The final complex of C5–9 (membrane attack complex) forms pores in the plasma membrane causing cellular lysis. Modified after Abbas AK, Lichtman AH, Pober JS. *Cellular and molecular immunology*, 4th edn, Saunders, Philadelphia, 2000; Carroll MC. The complement system in regulation of adaptive immunity. *Nature Immunology* 2004, 10: 981–986, with permission.

the outer leaflets of bacterial membranes, followed by the insertion of the hydrophobic part of the peptide into the lipid bilayer of the bacterial membrane, resulting in disruption of the bacterial membrane. Conversely, the outer leaflet of animal cells carries little net charge thereby avoiding the initial binding of amphipathic peptides.

There are three subfamilies of defensins, α-defensins found in neutrophils, β-defensins found in epithelial cells and θ-defensins (neutrophils of old world monkeys). In contrast to the antibacterial action of defensins, the mechanism of their antiviral activity is less clear, perhaps because it is pleiotropic. Some members of the defensin family prevent entry of several herpesviruses, either by preventing attachment to host cells or by blocking post-attachment steps in the entry pathway. CD8 T lymphocytes express α-defensins and these have been shown to be active against HIV-1, although it is controversial whether they are responsible for the anti-HIV activity of CAF (CD8-associated factor).

Complement and 'natural' antibodies

The serum of a number of mammalian species exhibits non-specific antiviral activity which may be mediated by either complement or by 'natural' antibodies. The complement system consists of a group of proteins that circulate in the plasma. As shown in Figure 5.7, these proteins can participate in a biochemical cascade that results in the production of channels in a lipid bilayer, resulting in lysis of viruses or other foreign pathogens. Complement acts as part of the effector mechanism for an induced immune response (described in Chapter 6). However, complement may also act as a non-specific host defense against certain viruses. For instance, a number of animal retroviruses are lysed by human complement in the absence of antiviral antibody. Apparently, p15e, one of the virus encoded proteins on the surface of some groups of retroviruses, binds C1q, one of the complement proteins, thereby activating the complement cascade. Complement-initiated lysis in the absence of specific antibodies has also been reported for some other viruses.

'Natural' antibodies are found in sera of many animal species and often are directed against foreign antigens to which humans or animals may be exposed. One example is antibodies against a disaccharide consisting of two galactose molecules bound in an α1-3 linkage. This digalactose is not synthesized by humans or other old world primates who lack a galactosyl transferase specific for the α1-3 bond. Since old world primates are exposed to this disaccharide present on ingested proteins, they develop anti-galactose (α1-3) antibodies. Viruses grown in cells that express the digalactose incorporate this molecule into the carbohydrate sidechains of their surface proteins. Such virions are neutralized by sera from old world primates that have never been exposed to the virus itself.

Other intrinsic host defenses

Animals have evolved a number of non-immune mechanisms which are used to contend with unwanted viral invaders. Since these do not involve acquired immunity, they could be considered 'intrinsic' host defenses, although

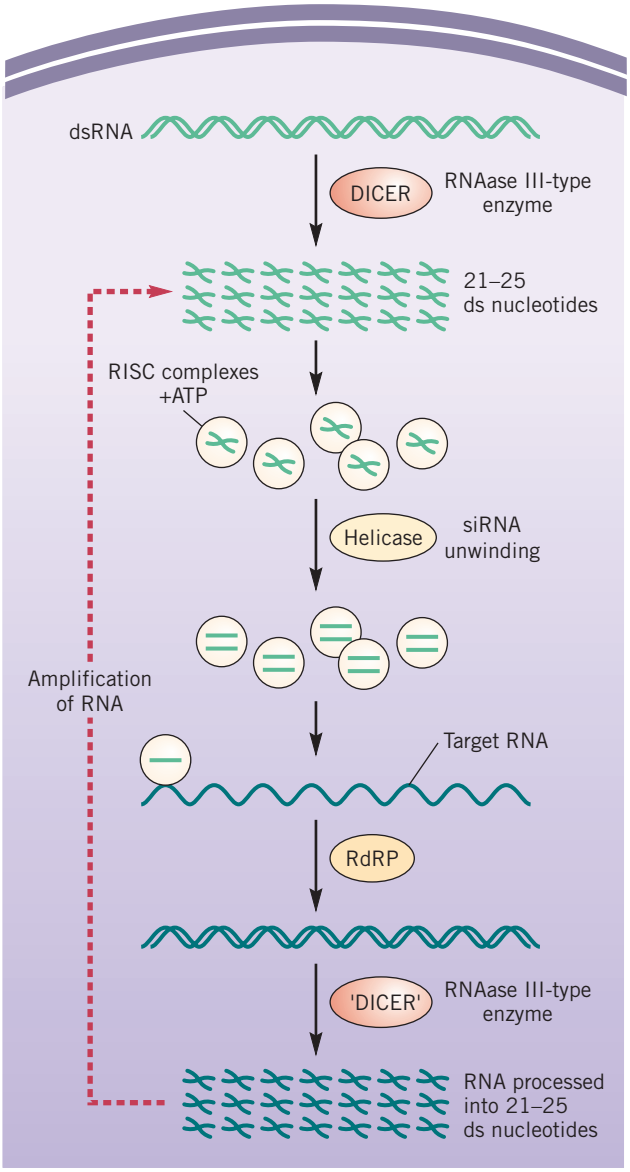

FIGURE 5.8 Diagram of RNAi (RNA interference). Synthesis of double-stranded RNA (dsRNA) is an essential step in the replication of most viruses. dsRNA triggers the action of an RNAse III-type enzyme (homolog of the Drosophila enzyme 'DICER') that cleaves the dsRNA into ds21–25 nucleotides. These oligonucleotides act as small interfering RNAs (siRNAs) by forming RNA-induced silencing complexes (RISCs) which unwind the siRNAs into single stranded molecules. These ssRNAs guide the complex to bind to homologous ssRNAs which are then converted into dsRNAs by RNA-dependent RNA polymerase (RdRP); the nascent dsRNA is then degraded by DICER homologs, effectively silencing the corresponding viral gene. After Dave RS, Pomerantz RJ. RNA interference: on the road to an alternate therapeutic strategy! *Reviews in Medical Virology* 2003, 13: 373–385, with permission.

they fall outside the usual definition of innate immunity. A few examples are described below.

RNA interference (RNAi)

In the mid-1990s, RNA interference was discovered serendipitously when an attempt to over-express specific plant genes, using viral vectors, instead resulted in the knockout or silencing of those genes. The mechanism has now been elucidated to some extent (Figure 5.8). Double-stranded RNA is synthesized as an essential intermediate in the replication of most viruses. Eukaryotic organisms have a ribonuclease III (class of ribonucleases specific for double-stranded RNA) that cleaves dsRNA into small ds oligonucleotides of 21–25 base pairs (the first member of this enzyme family was described in Drosophila and named 'DICER'). DICER and its homologs act to protect host cells against foreign RNAs, including those generated by invading viruses. The oligonucleotides produced by DICER homologs act as small interfering RNAs (siRNAs) by forming RNA-induced silencing complexes (RISCs) which, with the participation of helicase, unwind the ds oligonucleotides. The resultant ss oligonucleotides guide the RISC to homologous RNAs, which are then degraded.

RNAi has been adapted for the experimental silencing of specific genes. Using cell cultures, siRNA introduced by a variety of methods (such as expression by a lentivirus vector) has the ability markedly to reduce replication of HIV-1 and other viruses. Is RNAi used by host cells as an 'innate' antiviral defense? Recent studies demonstrate that RNA silencing is used by human T lymphocyte cell lines as a defense against HIV-1.

APOBEC3G

The APOBEC family of cellular proteins are cytidine deaminases. APOBEC3G associates with nascent HIV nucleocapsids and is incorporated into new virions. When these virions infect a new host cell and the viral reverse transcription complex is formed, APOBEC3G deaminates cytidine to uridine; the dU-rich DNA transcripts are either degraded or form hypermutated defective proviruses. The APOBEC family of deaminases serves as a host defense against retroviruses, hepadnaviruses (hepatitis B virus) and, perhaps, other retro-elements. The Vif protein of HIV-1 provides a mechanism for escape from this host defense by targeting APOBEC3G for the ubiquitination pathway (see Chapter 4 for a more detailed description).

RELATIONSHIP BETWEEN INNATE AND ACQUIRED IMMUNE RESPONSES

Innate and acquired immune responses act in a synergistic manner to defend the host against viral infection (Table 5.4).

Filling the gap between viral invasion and acquired immunity

Innate responses can be activated within hours of invasion, while days to weeks are required for induction of acquired responses. Acquired responses are potentially more critical because they have a much narrower focus on epitopes specific for an individual virus, but this requires a vast expansion of a small number of clonal precursor cells that inevitably takes considerable time. The sequence of innate and acquired responses is shown in Figure 5.9 for a model virus infection. For an acute viral infection, typically it requires about one week for the induction of acquired responses. During this interval, NK cells are activated and high levels of

Property	Innate	Acquired
Specificity	Broad PAMP-based	Fine Epitope-based
Induction time	Hours	Days to months
Requires antigen processing	No	Yes
Involves clonal expansion	No	Yes
Responder cells	Macrophages, DCs, NK cells	CD4+, CD8+ T cells, B cells
Effector cells	DCs, NK cells	CD8+T cells (CD4+T cells)
Effector mechanisms	Phagocytosis, cytolysis by NK cells, chemotaxis, cytokines, chemokines	Antibodies, cytolysis, cytokines, chemokines
Memory and effector recall	No	Yes
Long-term persistence	No	Yes

TABLE 5.4 Comparison of innate and acquired immunity
DC: Dendritic cells; CD: cluster of differentiation; NK: natural killer.

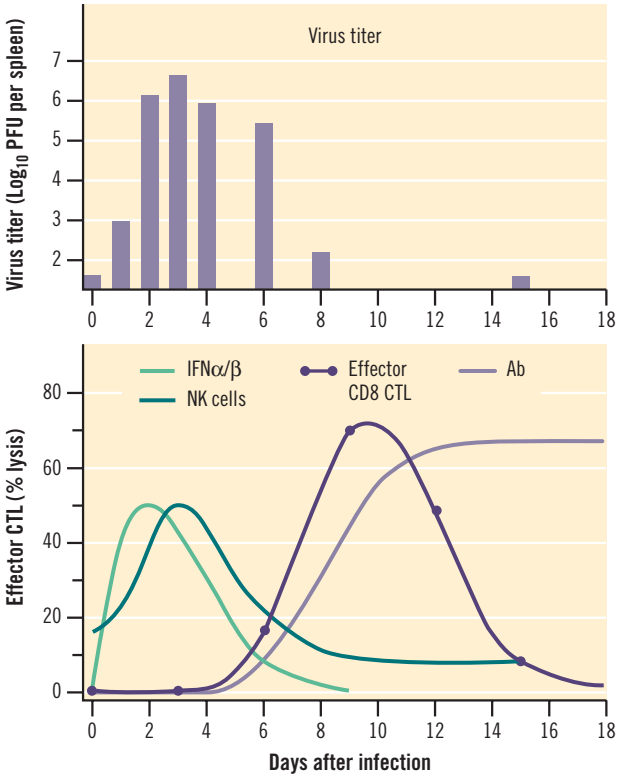

FIGURE 5.9 The non-specific responses to an acute viral infection precede the induction of antigen specific responses. This diagram shows the sequence of IFN α/β levels, NK cell activity, virus-specific CTLs and serum antibody (Ab) responses during acute infection with lymphocytic choriomeningitis virus. The IFN, NK and Ab curves are drawn to an arbitrary scale. After Welsh RM. Natural killer cells in virus infections. *Current Topics in Microbiology and Immunology* 1981, 92: 83–106; Biron CA. Cytokines in the generation of immune responses to, and resolution of, viral infections. *Current Opinions in Immunology* 1994, 6: 530–538; Lau LL, Jamieson BD, Smasundarma T, Ahmed R. Cytotoxic T cell memory without antigen. *Nature* 1994, 369: 648–652, with permission.

type 1 IFN (α and β) are secreted. These responses hold the infection in check during the induction of specific acquired immunity. The critical role of innate immunity is demonstrated in experimental models in which abrogation of specific components of the innate response potentiates viral infection (see Figures 5.3 and 5.6).

Innate response prepares for induction of acquired immunity

Certain components of the innate response play a dual role since they constitute the first steps in induction of antigen-specific immunity. Typically, at the site of viral invasion, DCs bind and recognize PAMPs, mainly ssRNA and dsRNA in the case of viruses. This leads to activation of DCs, which secrete pro-inflammatory cytokines such as TNFα, IL-6 and IL-12; the inflammatory focus is a nexus that concentrates cells of the innate system. Activated DCs then migrate from their tissue locations via afferent lymphatics to T cell zones in draining lymph nodes (see Figure 5.2). Meanwhile, DCs are processing antigen, via two separate pathways, for presentation on class I and class II MHC (major histocompatibility complex) molecules (discussed in Chapter 6). In addition, there is an upregulation of CD80 and CD86 molecules that provide a 'second signal' essential for activation of antigen-specific T lymphocytes. Also, it appears that activated DCs play a role in over-riding the potential suppression of Th cells by regulatory T (Tr) lymphocytes.

The role of the innate response in induction of acquired immunity has been demonstrated in a variety of approaches, including studies of humans with specific genetic defects, knockout mice that lack individual genes involved in the innate response, antibody-mediated depletion of specific cell surface receptors, or variant viruses that can evade innate immunity. A particularly significant example of the latter is the study of H5N1 avian influenza viruses that infect humans rarely, but produce a high mortality (often over 25%). In this instance, it appears that the NS1 (non-structural) gene of the H5N1 virus renders it resistant to IFN, which accounts for its high virulence in mammalian hosts (Table 5.5).

REPRISE

Beginning around 1990, innate immunity was 'rediscovered' as an important component of host defense against infection. Innate immunity is the only host defense system in non-vertebrate animals and synergizes acquired immunity in vertebrates. Macrophages, dendritic cells (DCs) and NK cells are mainly responsible for innate responses, but the important innate cytokines, type 1 IFNs, can be produced by virtually any nucleated cell if it is appropriately stimulated. Macrophages and DCs carry

	Influenza viruses		
	Reference H1N1 mammalian	Virulent H5N1 avian	Recombinant RecH1N1-NS1/H5N1
Replication in cell culture at 72 hours (log_{10} TCID50 per ml)			
Untreated	7.25	6.25	4.6
Pretreated with IFNα	<1.0	6.5	4.4
Infection of pigs			
Duration of virus replication (nasal swabs)	4 days	ND	8 days
Weight at 14 days after infection, as percent of initial weight	105%	ND	60%

TABLE 5.5 The NS1 gene of virulent avian influenza virus H5N1 confers resistance to IFNα and virulence in mammalian hosts. This table compares three influenza viruses, avian virus H5N1 (H5N1), reference mammalian virus H1N1 (H1N1) and a recombinant H1N1 virus with the NS1 gene of H5N1 (RecH1N1-NS1/H5N1). Compared to reference mammalian viruses such as H1N1, the avian H5N1 virus is extraordinarily resistant to INFα and is more virulent following intranasal infection of pigs (an important natural host for influenza viruses)
After Seo SH, Hoffman E, Webster RG. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. *Nature Medicine* 2002, 8: 950–954.

pattern recognition receptors (PRRs) that respond to pattern associated molecular patterns (PAMPs), motifs common to large classes of infectious agents but often absent in eukaryotic organisms. For viruses, ssRNA and dsRNA, distinct from normal cellular RNAs, are the major PAMPs. Once PRRs have been ligated, they set off intracellular biochemical cascades that lead to cellular activation. Activated cells initiate phagocytosis, chemotaxis and the secretion of many cytokines and chemokines, such as interferon type I and α-defensins, in turn, induce inflammation and other antiviral responses. A new class of cytoplasmic receptors may function to detect virus in infected cells and induce IFN production by a wider range of cell types. Innate immunity synergizes with acquired immunity in several ways. It is initiated within hours and provides a rapid array of defenses while antigen-specific immune responses are being induced during the first weeks after infection. Furthermore, selected innate responses – such as the activation of DCs, initiation of antigen processing, migration to draining lymph nodes and upregulation of co-stimulatory molecules – are the essential first steps in induction of acquired immunity.

FURTHER READING

Books, chapters and reviews

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Chapter 6

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ACKNOWLEDGEMENT

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Immune Responses to Viral Infection

Neal Nathanson and Rafi Ahmed

SPECIFIC AND INNATE IMMUNE RESPONSES TO VIRAL INFECTION

Following viral invasion, the infected host mounts a number of responses to infection. Many of these responses involve the induction by the adaptive immune system of antibodies or cells that are specific for antigenic determinants expressed by the foreign pathogen. Most of this chapter is devoted to a description of these specific immune responses. Infected hosts also possess a number of non-specific defenses against an invading virus and these have been discussed in Chapter 5, which deals with innate immunity. In general, innate defenses come into play at the time of infection or shortly thereafter, filling an important gap in protection against viral invasion, until the appearance of specific immune responses that characteristically require days to weeks for induction.

OVERVIEW OF THE IMMUNE SYSTEM

The following cursory description is designed to refresh the reader's memory about the organization and function of the immune system. Students who are not familiar with basic immunology may wish to consult one of the many excellent introductory texts (see Further reading).

The players: cells of the immune system

Lymphocytes are responsible for both the induction and expression of cellular immunity. There are two major classes of lymphocytes, B cells and T cells. T cells are named after the thymus, while B cells are named after the Bursa of Fabricius, an organ found in birds (but not in mammals) where B lymphocytes undergo early maturation. In mammals, the same steps in B cell maturation occur in the bone marrow.

All types of lymphocytes are constantly produced from precursor stem cells residing in the bone marrow. Progenitor cells migrate from the bone marrow to the thymus where they undergo maturation that prepares them to respond to an immune stimulus; they emerge from the thymus as 'naïve' T cells that migrate to spleen, lymph nodes and selected tissues such as the skin and submucosal sites. B lymphocytes emerge from the bone marrow and migrate directly to lymphoid tissues.

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The other important cells involved in immune responses are monocyte/macrophages and dendritic cells. Monocytes arise from stem cell precursors in the bone marrow and leave the bone marrow to circulate as the blood monocyte, following which they may enter tissues to reside as tissue macrophages. Dendritic cells have several lineages (see Figure 5.2) and are found in skin and mucosal tissues (and in lymph follicles) where they specialize in the binding of foreign antigens and the initiation of immune induction. Professional antigen presenting cells are specially equipped to initiate immune induction, since they express both MHC class I and class II molecules. Lymphoid tissues, such as the spleen and lymph nodes, have a rather complex structure that is beyond the scope of this chapter. Suffice it to say that macrophages, dendritic cells and lymphocytes are situated within spleen and lymph nodes at locations that optimize their ability to play specific roles in response to immune stimuli.

Each immune cell bears molecules on its surface that equip it to discharge its specialized role in the immune response (Table 6.1). B cells carry antibody molecules that bind 'epitopes' or individual immune determinants on foreign antigens. Antigens recognized by B cells may be either proteins, carbohydrates, or nucleic acids and their epitopes consist of a small cluster of amino acids, sugars, or nucleic acids, respectively. Thus, an epitope would include about 10 amino acids, while a protein could be composed of 25–1000 amino acids. Antibodies can recognize both linear and conformational epitopes in proteins.

T lymphocytes carry a T cell receptor (TCR) that is a heterodimer composed of an α and a β-polypeptide. T cell receptors have a structure that is roughly analogous to the antibody molecule but, in contrast to antibodies, recognizes amino acids within a peptide of 8–11 amino acids bound to a specialized groove on the surface of an MHC class I molecule (10–30 amino acids for MHC class II molecules). Individual B and T cells express different antibodies or TCRs, specific for individual epitopes of different antigens. These different receptors are 'constructed' by rearrangements of genetic determinants expressed in germ line cells, rearrangements that occur during maturation of lymphocytes. Due to this exquisitely specialized developmental arrangement, lymphocytes can encode up to $\sim 10^9$ different specificities. In contrast to lymphocytes, macrophages or dendritic cells are equipped with more general receptors for the Fc component of antibody molecules and with other surface molecules that enable them to bind and internalize whole foreign antigens.

T lymphocytes can be divided into two major categories, CD4+ and CD8+ cells, which subserve different immune functions. These two cell types can be identified by the expression on their surface of either CD4+ or CD8+ molecules, which are used as markers for purposes of enumeration or cell sorting. CD4+ cells carry TCRs that recognize antigen presented by MHC class II but not class I molecules and, conversely, CD8+ cells recognize antigens presented by class I molecules.

CD4+ cells act as 'helper' cells by interacting with naïve B lymphocytes or CD8+ lymphocytes to provide signals that induce B cells or CD8+ cells to proliferate in response to an antigen presented by a professional antigen-presenting cell (APC); dendritic cells, macrophages and B cells can act as APCs. The signals provided by CD4+ helper cells involve ancillary surface molecules that interact with cognate ligand molecules on the surface of the antigen-responding CD8+ or B cells. Thus, there are two interactions involved in optimal immune induction, the recognition by a very small subset of lymphocytes of an epitope that is cognate to its recognition molecule (antibody or TCR) and the non-specific second signal that initiates clonal proliferation of such antigen specific lymphocytes.

CD4+ cells can be divided into T_H1 and T_H2 cells. T_H1 cells secrete large amounts of IFNγ and IL-2 and drive the clonal expansion of CD8+ cells; recent studies suggest that there may be a subclass of T_H1 cells that secrete IL-17 (T_H-17 cells). T_H2 cells secrete large amounts of IL-4, IL-5 and IL-21 and drive the clonal expansion of B cells.

CD8+ lymphocytes are effector cells that mediate the cellular immune response by their ability to recognize foreign epitopes presented by class I molecules. CD8+ cells initiate a local response that includes two components, a lytic attack on target cells carrying a foreign epitope and the production of cytokines that can

	B cell response	T cell response	T cell response
Type of immunity	Humoral	Cellular	Cellular
Precursor (memory) cell	B lymphocyte	CD4+ precursor	CD8+ precursor
Effector cell	Plasma cell	CD4+ lymphocyte	CD8+ CTL
Mediator molecules	Immunoglobulins (Igs)	Cytokines	Perforins, granzymes, cytokines
Persistence of effectors	Yes	No	No
Anamnestic (memory) response	Yes	Yes	Yes

TABLE 6.1 Comparison of B and T cell immune responses
CTL: cytolytic T lymphocyte.

attract inflammatory cells to the local site, resulting in an indirect attack on the invading parasite. Individual CD8+ cells differ in their ability to mount these two responses and the balance may be influenced by the conditions of immune induction.

Induction of the immune response

Immune induction of antibody and of cellular immunity involves different cell types. The B lymphocyte pathway (Figure 6.1) begins with the binding of foreign proteins to the immunoglobulin molecules expressed on the surface of naïve B cells. Naïve B cells are already programmed genetically to express Ig molecules with a single antigenic specificity, i.e. they will recognize only a single epitope of the multitude of determinants on the foreign protein. Meanwhile, dendritic cells and macrophages ('professional' APCs) bind the same foreign protein. Foreign proteins are endocytosed, digested to oligopeptides and 'loaded' onto class II MHC molecules on the surface of APCs. CD4+ T_H2 lymphocytes, whose T cell receptors (TCR) recognize the specific peptide presented by the

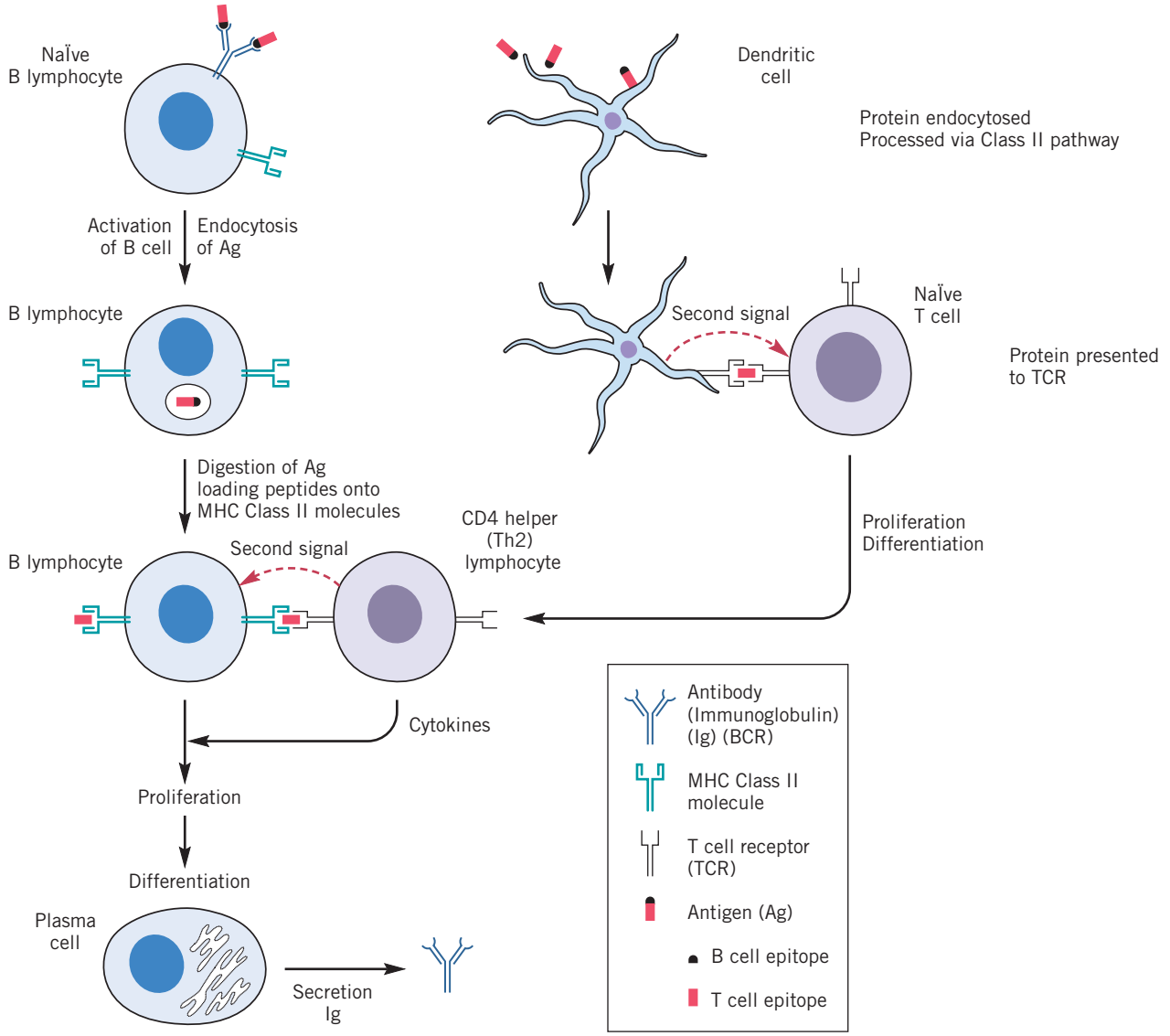

FIGURE 6.1 A simplified representation of induction of a B lymphocyte (antibody) response showing the major cell types involved. (1) A naïve B lymphocyte binds a protein antigen via its surface immunoglobulin (Ig) (B cell receptor, BCR) that recognizes a specific epitope on the protein, resulting in activation of the B cell through the B cell receptor. The protein that binds to and stimulates the BCR can also be endocytosed and processed through the exogenous MHC class II pathway and the B cell presents (on its surface) a peptide (different from the epitope that binds to the BCR on its surface) bound to its class II molecules. (2) Dendritic cells endocytose the protein, process it through the class II pathway and present peptides bound to MHC class II molecules. The peptides are recognized by a subset of naïve CD4+ T lymphocytes bearing the cognate T cell receptors (TCRs) which then proliferate, differentiate and act as helper cells (CD4+ T_H2 cells). (3) CD4+ T_H2 cells recognize the peptides presented by B cells on their class II MHC molecules and stimulate B cells in two ways. First, CD4+ cells provide a second signal via the CD40-CD40 ligand pathway; CD40 ligand on CD4+ cells binds CD40 on B cells, initiating B cell intracellular signaling. Second, CD4+ cells secrete cytokines (IL-2, IL-4, IL-5) that stimulate the presenting B lymphocytes to proliferate and differentiate into end stage plasma cells that secrete their epitope-specific Ig molecules. Antibodies are continuously produced, even in the absence of antigen, both by long-lived plasma cells in the bone marrow and possibly by continual differentiation of memory B cells into mature plasma cells.

class II molecules on the APCs, and B cells will be stimulated to release cytokines (IL-4, IL-5, IL-21) that drive the B cells to proliferate and differentiate. Mature B cells (plasma cells) will then secrete the immunoglobulin for which they have been programmed.

The cellular immune response follows a stereotyped sequence of events (Figure 6.2), although there are many variations. If a foreign antigen, such as a virus protein, is introduced via any portal of entry, some of the molecules are bound by professional APCs. The dendritic cell can process protein antigens by two pathways, an exogenous and an endogenous pathway. The endogenous pathway may be utilized if the virus can infect the APC and begins with the expression of a foreign protein in the cytosol of the professional APC. Viral proteins synthesized in the cytosol of the APC are delivered to the proteosome, a complex cytoplasmic organelle, which digests the protein into peptides and delivers these peptides (mainly 9–11 mers) across internal membranes into the endoplasmic reticulum, where they are loaded onto class I molecules. The exogenous pathway involves endocytosis of the protein, digestion into small oligopeptides in endolysosomes and 'loading' of these 10–30 mers onto the antigen binding groove on class II molecules. Some class I molecules can also be loaded via the exogenous pathway, a phenomenon known as 'cross presentation'.

Once exposed to foreign antigens in the periphery, dendritic cells migrate rapidly via afferent lymphatic channels to draining lymph nodes. Within lymphoid tissue, dendritic cells interact with CD8+ cells whose TCRs

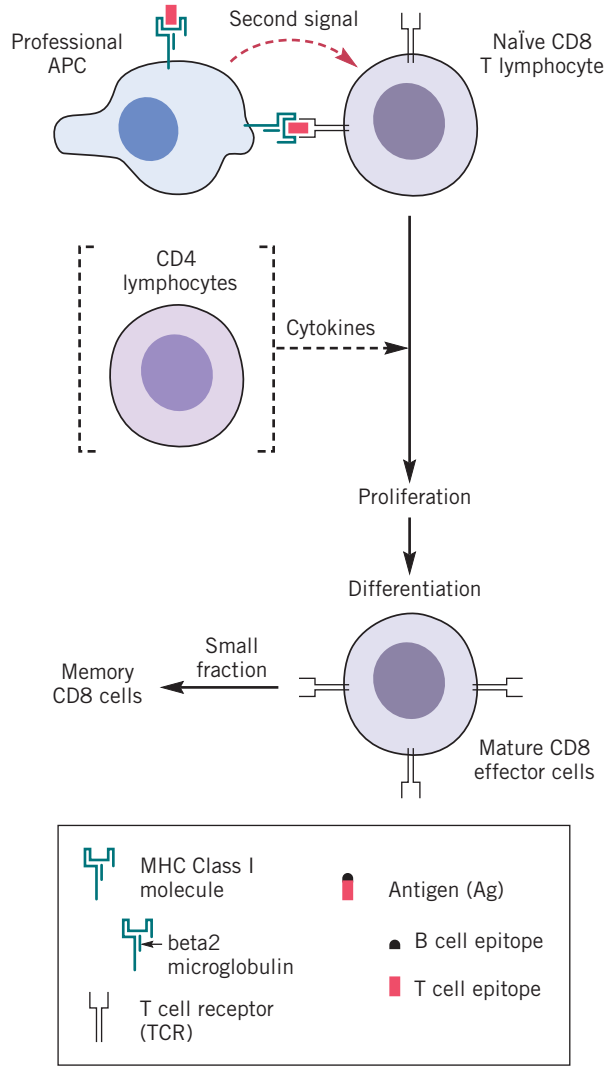

FIGURE 6.2 A simplified representation of induction of a cellular (CD8+ T cell) immune response showing the major cell types involved. A professional antigen presenting cell (APC) bears MHC class I molecules that present a specific peptide bound to the antigen binding groove. Naïve CD8+ T lymphocytes expressing a T cell receptor (TCR) that recognizes the same peptide will bind to the peptide and be stimulated to proliferate. Proliferation is triggered by two 'signals', the recognition of the peptide and by cross linking of interactive accessory proteins on the surfaces of APCs and naïve T cells (such as CD40-CD40 ligand). The ability to provide this second signal distinguishes professional APCs from most other cells that express class I molecules. Following proliferation, CD8+ lymphocytes differentiate into mature CD8+ lymphocytes that are capable of recognizing and 'killing' target cells bearing the same epitope on their class I molecules. For many viruses, CD4+ helper T lymphocytes (via cytokines such as IL-2 and IL-4) appear to play a limited role in induction of effector CD8+ cells, but they are more important for the generation of CD8+ memory cells derived from the CD8+ effector cell pool.

recognize the epitopes presented by the class I molecules on professional APCs. If they receive a 'second signal' provided by dendritic cells or by CD4+ cells, antigen-responsive CD8+ cells then undergo clonal expansion and differentiate into effector CD8+ cells (Figure 6.2). CD4+ helper cells play a variable role in CD8+ induction and are most important for the generation of the memory subset (rather than the effector subset) of CD8+ responding lymphocytes. Thus, HIV infects and kills CD4+ cells, but indirectly reduces the ability of CD8+ effector cells to contain longstanding persistent opportunistic infections, a phenomenon that is discussed in Chapter 14.

A virus infection will simultaneously induce antibody and a cellular immune response. The balance between the two responses will be influenced by the relative proportion of T_H1 and T_H2 cells participating in the immune response. As noted above, the effect of T_H1 and T_H2 cells is mediated by the cytokines that they secrete – IFNγ, IL-2 and IL-17 driving CD8+ cells, while IL-4, IL-5 and IL-21 drive B cells.

Genetic approaches to studying immune responses

Beginning around 1990, a series of methodological advances were introduced that have made it possible to manipulate the mouse genome so as to insert or delete individual genes (see Further reading for more detailed descriptions). In turn, this has led to a revolution in immunobiology, since it has become possible, in a whole animal, to probe the specific role of each of the vast array of genes that control the development and function of the immune system. These advances are now being used to analyze the immune response to viral infections in much more detail than was possible in the past. Experiments employing genetically modified mice are cited frequently in the rest of this chapter.

Methods that are frequently used to manipulate the activity of individual genes include:

- Transgenes. The DNA sequence of a known gene is engineered to introduce a desired change in its sequence that is randomly integrated into the recipient DNA by non-homologous recombination, so that it does not replace the corresponding normal gene.
- 'Knockout'. The DNA sequence of a known gene is engineered to introduce an alteration that renders it non-functional. The 'knockout' construct undergoes homologous recombination so that it substitutes for and 'knocks out' the target gene.
- 'Knockin'. The same methods used to produce 'knockout' mice are used to introduce an engineered gene in place of its normal counterpart.
- Inducible 'knockout'. The introduced 'knockin' target gene construct includes an additional sequence (such as loxP in the loxP-Cre recombination system) and a gene (Cre, a site-specific recombination enzyme) that can be induced to 'knockout' the target gene.
- Small interfering RNA (siRNA). A double-stranded siRNA is synthesized with a sequence identical to a sequence within the target mRNA. When introduced in vivo, the siRNA can lead to the destruction of the target mRNA and reduce expression of the corresponding protein (see Chapters 5 and 16 for further discussion of siRNA).

ANTIBODY

Measurements of antibody

There are many methods to measure antibody to viruses and both the kinetics of the response and its biological significance depend upon the assay used. The canonical assay is the neutralization test, in which antibody is tested for its ability to reduce viral infectivity. This test depends on the availability of a convenient method to measure viral infectivity, often a plaque assay, in which each infectious virus particle produces a single lytic plaque in a lawn of susceptible indicator cells. One common technique involves the use of a single viral inoculum, such as 100 PFU (plaque forming units); serial dilutions of a test antibody are tested to determine the highest dilution that will reduce the plaque count by 50% (Figure 6.3). An alternative less frequently used method is the neutralization index, in which the titer of a virus stock is compared in the presence and absence of a test antibody; the index is calculated as the difference in viral titers. Neutralization tests cannot be used for some important viruses, such as hepatitis B and C viruses, that cannot readily be grown in cell culture.

There are many alternative assays that measure the ability of the antibody to bind to viral antigens, including hemagglutination inhibition, immunofluorescence, Western blot and ELISA (enzyme linked immunosorbent assay). Of these, the most commonly used is the ELISA assay, which can readily be adapted to quantitation, automation and rapid throughput. An antigen, either whole virus, a viral protein or a viral peptide, is bound to a substrate and then incubated with serial dilutions of test antibody; adherence of the test antibody is determined with a conjugated antiserum directed against immunoglobulin of the species under test. The conjugate may carry a fluorescent or other visualizable label or be an enzyme that will convert a substrate from a colorless to a visible form.

In contrast to the ELISA, the Western blot is a qualitative test that provides information about the specificity of the test antibody (Figure 6.4). Proteins from a viral lysate are separated, often using polyacrylamide gel electrophoresis, and crosslinked to a cellulose strip. An unknown serum is tested for its ability to bind to any of the proteins on the strip and the reaction is 'developed' with a labeled antisera against immunoglobulin, as for the ELISA.

Antibody production can also be measured at a cellular level, although this is rarely done except for special experimental purposes. In the ELISPOT assay, cells – including plasma cells – are prepared from blood or lymphoid tissues and overlaid on a surface to which a target antigen has previously been bound. Antigen-specific antibody released by specific plasma cells binds to the cognate antigen and the 'focus' is developed using a variation of the method used in ELISA assays. This assay

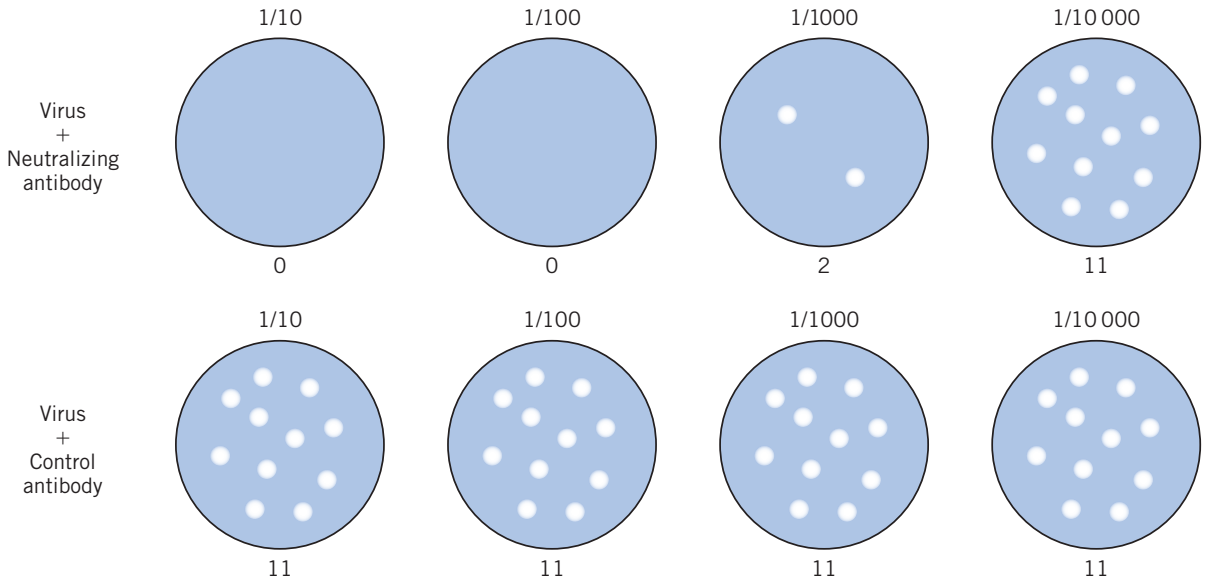

FIGURE 6.3 A plaque reduction neutralization test showing the ability of a test antibody to reduce the infectivity of the virus (for convenience of illustration, ~10 PFU are shown, although a larger inoculum is usually employed). The 50% endpoint is calculated as the dilution of antibody that would reduce the plaque count from the control level of 10 to 5 PFU. In this example the endpoint is ~1/3750.

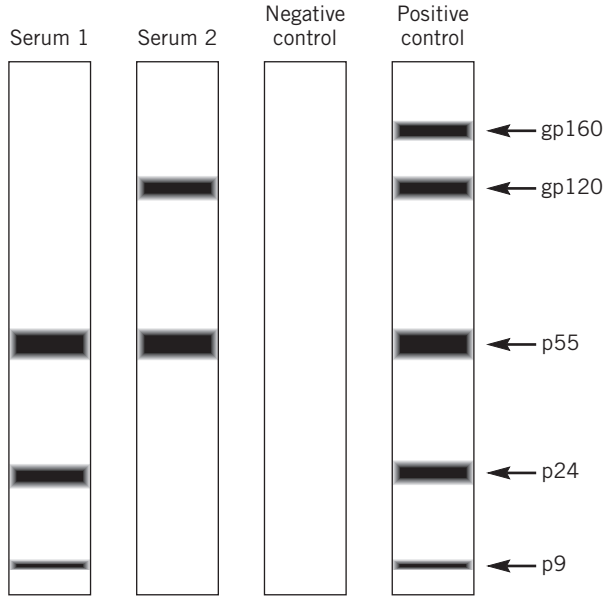

FIGURE 6.4 Western blot test for antibody. Four sera are compared, two containing antibody against viral antigens and a positive and negative control. The positive serum reacts with all of the viral proteins on the test strip, while each of the test sera shows a different pattern of reactivity.

permits the counting of antibody-secreting cells (ASCs) and can be employed for studies of the dynamics of the antibody response.

Effector functions of antibody

Antiviral antibodies can subserve host defenses in several different ways. First, they can bind to virus and neutralize it, i.e. render the virion non-infectious. There are multiple mechanisms of neutralization, but the most common is to coat the virion and prevent its attachment to receptors on permissive host cells. A minority of neutralizing antibodies bind to the virus but do not prevent attachment to the cell; instead they interfere with later steps in viral entry. Also, it should be noted that many antibodies that bind to viral proteins fail to neutralize, usually because the specific epitope is not expressed on the surface of infectious virions – for instance, if the epitope is part of an internal protein not present on the surface of the virion.

In addition to reducing infectivity of virions, antiviral antibodies can act in several other ways. Many antibodies will bind C1q, the first of the complement proteins, and initiate lysis of the virion. Also, many antibodies will bind to the Fc receptors on Kupffer cells and other sessile macrophages. Complexes consisting of virions bound by antibodies will be more efficiently phagocytosed and degraded than free virions and this process is sometimes called opsonization. Finally, antibodies can 'arm' NK cells that then mediate ADCC (antibody-dependent cell-mediated cytolysis) leading to the destruction of infected cells. NK cells carry receptors for the Fc domain of immunoglobulin molecules. If an antibody with virus specificity binds to the FcR of an NK cell and the NK cell then contacts a virus-infected cell bearing the cognate antigen, it can initiate a cytolytic attack similar to that mediated by CD8+ cytolytic T cells.

In general, the ability of antibodies to protect in vivo correlates with their neutralizing activity. However, this correlation is not absolute, as shown in studies of individual monoclonal antibodies (Table 6.2). The mechanism whereby non-neutralizing antibodies protect is not well understood but, in some instances, may be mediated by the clearance of virus-antibody complexes by the reticuloendothelial system.

Neutralization in vitro	Protection in vivo (number of monoclonal antibodies)	
	Yes	No
Yes	2	1
No	3	3

TABLE 6.2 The ability of monoclonal antibodies to neutralize in vitro does not necessarily correlate with their ability to protect in vivo. In this example, monoclonal antibodies (100 μg per animal) were administered to mice one day prior to injection of Ebola virus (10 PFU, a 100% lethal dose). Protection: from 6 to 10 mice survived challenge (of 10 tested); no protection: 0/10 mice survived. Neutralization required the addition of complement
After Wilson JA, Hevey M, Bakken R *et al.* Epitopes involved in antibody-mediated protection from Ebola virus. *Science* 2000, 287: 1664–1667, with permission.

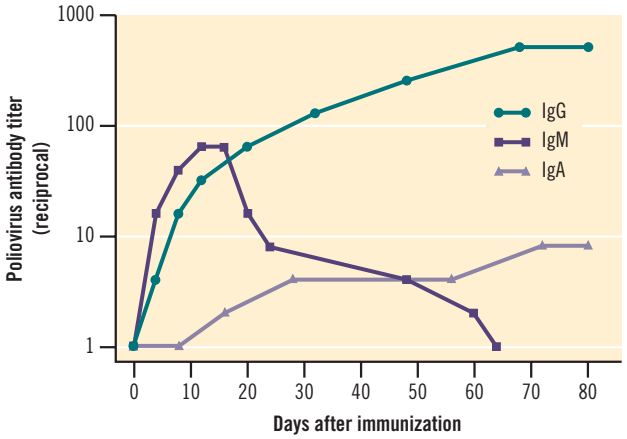

FIGURE 6.5 The time course of different classes of antibody response. Subjects were immunized with poliovirus vaccine (IPV, inactivated poliovirus vaccine or OPV, oral poliovirus vaccine) at 0, 32 and 56 days and followed for their titers of serum antibody (IgG,, IgM, IgA). The IgM response is rapid but transient while IgG and IgA are induced more slowly but are long lasting. After Ogra PL, Karzon DT. Formation and function of poliovirus antibody in different tissues. *Progress in Medical Virology* 1971, 13: 156–193, with permission.

Kinetics of the antibody response

Antibodies can be measured in different bodily fluids, such as blood (plasma or serum) and mucosal fluids (nasal, throat, bronchial washes, feces, semen, genital washes). Furthermore, the class of antibody varies. Serum contains IgG (the principal class), IgM and IgA, most of which is derived from plasma cells in bone marrow, lymph nodes and spleen. Mucosal fluids contain IgA (locally produced by plasma cells in mucosa-associated lymphoid tissues or MALT) and IgG (partly a transudate of serum IgG and partly produced in MALT).

An example of the humoral (serum) antibody response is shown in Figure 6.5, which illustrates that the dynamics of the responses are quite different, according to antibody class. In general, serum IgM appears rapidly (1–2 weeks) and disappears in about 3 months, while IgG appears more slowly (2–4 weeks), peaks at 3–6 months and then gradually wanes, although it may persist at sub-maximal levels for a lifetime.

The ELISPOT assay has been used to trace the location and kinetics of plasma cells (mature antibody-producing B cells). As shown in Figure 6.6, during the primary immune response, most antibody-producing plasma cells reside in the spleen but, following acute infection, they are mainly found in the bone marrow. During re-infection, there is another transient increase in plasma cells in the spleen as well as expansion of the continuing population in the bone marrow. The immune host continues to produce considerable titers of virus-specific antibody following primary infection, an important distinction from cellular immunity (CD8+ effector cells) that is not maintained at high levels after disappearance of the virus. Persistent antibody production is likely due to several mechanisms, including long-lived plasma cells in the bone marrow and (possibly) memory B cells that continue to differentiate into newly mature plasma cells, stimulated by antigen or by cytokines in the local environment. It is speculated that the bone marrow may provide a high concentration of cytokines that are essential for the survival of mature plasma cells and the continual proliferation of plasma cell precursors.

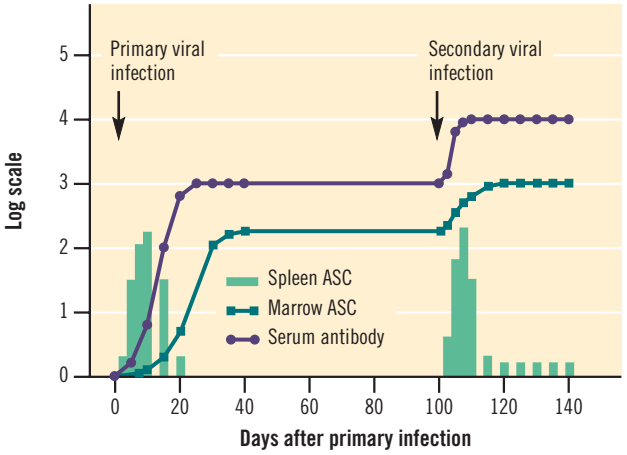

FIGURE 6.6 The time course and localization of plasma cells secreting virus-specific antibody. LCMV infection of the mouse induces the expansion of plasma cells (ASC, antibody-secreting cells) in the spleen during acute infection. With the waning of the infection, about one month later, the level of antibody production is maintained but is mainly due to plasma cells in the bone marrow. During re-infection, there is an increase in the level of serum antibody associated with a transient spurt in the number of plasma cells in the spleen. After Slifka MK, Matloubian M, Ahmed R. Bone marrow is a major site of longterm antibody production after acute viral infection. *Journal of Virology* 1996, 69: 1895–1902, with permission.

CELLULAR IMMUNITY

Measures of cellular immune response

Limiting dilution assay

The classical assay for cellular immune responses is the limiting dilution assay or LDA (Figure 6.7), introduced in the 1960s. Cells are obtained from blood, spleen or lymphoid tissues of an animal immune to the virus under study (virus 'X') and are cultured for 1–2 weeks in the presence of whole inactivated virus X, its proteins, or peptides. Under these conditions, virus X-specific

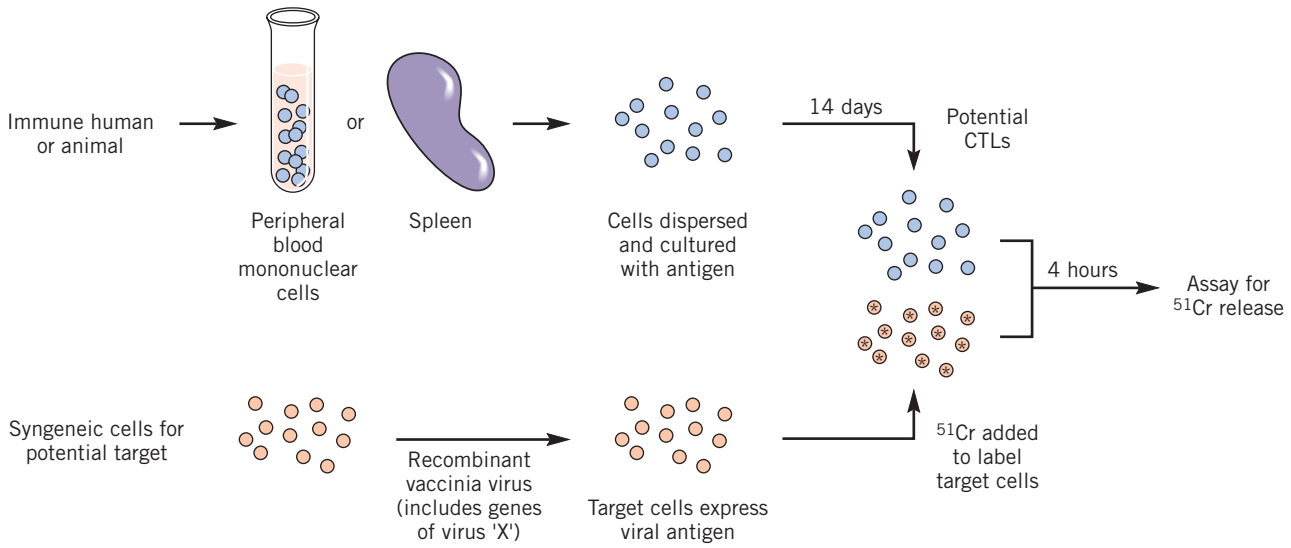

FIGURE 6.7 Measurement of CD8+ precursor cells based on their ability to lyse appropriate targets (the LDA or limiting dilution assay). CD8+ cells obtained from an immune donor are cultivated in the presence of the viral antigen under study, to expand the number of precursors and mature them into effector CTLs. Target cells are prepared from a syngeneic source, infected with a transformed vector so that they express the viral antigens on their surface and also labeled with ^{51}Cr as a marker of cell death. CD8+ cells are incubated with labeled target cells and the amount of lysis of targets is assayed by the degree of ^{51}Cr release. This diagrammatic representation does not show various controls and quantitative features of the assay.

CD8+ cells undergo clonal expansion, presumed proportional to their numbers in the original cultured sample. The expanded virus-specific CD8+ lymphocyte population is then tested for its ability to destroy target cells bearing the viral antigens in the context of syngeneic MHC class I molecules.

Production of syngeneic target cells depends upon the species. For mice, animals of a single inbred strain are used, while for outbred species, such as humans, target cells are usually prepared from each individual to be tested using EBV (Epstein-Barr virus) to immortalize B cells (B cells are used simply as targets). Target cells are then infected with a vector, such as a transformed vaccinia virus, that expresses the virus X antigens; the targets will now express the antigens of virus X bound to the subject's MHC class I molecules. The target cells are labelled with ^{51}Cr, a marker that binds to intracellular proteins. Cultured CD8+ cells are incubated with target cells and the release of ^{51}Cr is measured as an indicator that the targets have been lysed by the CD8+ effector cells. This assay can be quantified by serial dilution of CD8+ cells prior to the assay, yielding an estimate of the number of CTL precursors in the original cell suspension. Although the LDA represents a biologically relevant measure of cellular immunity, it is very cumbersome, tedious, capricious and expensive.

As described above, the CTL assay measures precursor (or memory) CD8+ cells, based on their potential for expansion in the presence of their cognate antigen. Alternatively, lymphocytes freshly obtained from blood or other tissues can be assayed for direct ex vivo killing, which is a measure of effector cells in contrast to precursor cells.

New assays for cellular immune responses have recently been introduced and are still under active development (Table 6.3). One group of assays is based upon the FACS (fluorescent activated cell sorter); FACS assays fall into two categories, tetramer assays and intracellular cytokine staining (ICS).

Tetramer assay

The tetramer assay detects antigen-specific CD8+ cells by a reagent that consists of a single peptide molecule bound in the antigen-binding groove of an MHC class I molecule that is chemically coupled into a tetramer that also bears a sensitive fluorescent marker. A tetramer reagent will bind to the surface of all CD8+ cells bearing TCRs specific for a single cognate peptide and such labeled cells can be enumerated using the FACS. It is assumed that numbers of tetramer-staining cells are proportional to the numbers of effector cells measured in the cytolytic assay.

Intracellular cytokine staining (ICS)

The other FACS-based assay is based on the response of T lymphocytes to the epitope recognized by their TCRs. Epitope exposure (oligopeptides added to the culture) stimulates the production of a number of interleukins and cytokines, such as IFNγ or TNFα (tumor necrosis factor). After epitope exposure, CD8+ cells are treated with brefeldin A (to prevent secretion of cytokines) and are subsequently fixed (killed with a chemical such as glutaraldehyde that leaves their structure intact). The cells are then permeabilized so their intracellular cytokine can be stained by a specific antibody carrying a fluorescent marker and the staining intensity corresponds to the level of cytokine expression. Lymphocytes can then be enumerated on a FACS to measure the particular intracellular cytokine. Using appropriate controls for calibration, it is now possible to determine the number of cells responding to a specific epitope in the context of a

Assay	Fresh or cultured cells	Target cell or marker	Readout
LDA (limiting dilution assay)	Cultured with antigen	Cell presenting epitope bound to syngeneic class I MHC	^{51}Cr release from target cells
CTL (cytolytic T cells)	Fresh or frozen; short-term culture with antigen	Cell presenting epitope bound to syngeneic class I MHC	^{51}Cr release from target cells
Tetramer	Fresh or frozen	Fluorescent complex with epitope bound to syngeneic class I MHC	FACS (fluorescence-activated cell sorter)
Intracellular cytokine staining (ICS)	Fresh or frozen	Fluorescent complex bound to intracellular cytokine (such as INFγ)	FACS (fluorescence-activated cell sorter)
ELISPOT	Fresh or frozen	Secreted cytokine (such as INFγ)	Monocellular focus of released cytokine in culture dish

TABLE 6.3 Measures of CD8+ T lymphocyte immune responses
Methods are described in Janeway CA, Travers P, Walport M, Shlomchik MJ. Appendix I, Immunologists' toolbox, in *Immunobiology*, 6th edn, Garland Science, New York, 2005.

specific MHC class I molecule. As with the tetramer assay, the numbers of cytokine-staining cells are probably proportional to the number of cells measured in the LDA. The ICS assay appears to be the assay of choice at this time (2005), particularly where sophisticated research equipment is available.

ELISPOT assay

A different method is the ELISPOT assay, which is similar to that described above for plasma cells. In this application, lymphoid cells are plated in medium and stimulated with oligopeptides. Those CD8+ T lymphocytes recognizing a specific epitope secrete cytokines. The surface of the culture plate has been prepared by coating with antibody that will recognize a particular cytokine, such as IFNγ and the number of foci of secreted and bound cytokine are then enumerated to count the number of responding CD8+ lymphocytes. The ELISPOT assay is often used for high throughput of large numbers of samples or where laboratory facilities are limited.

Effector functions of CD8+ lymphocytes

CD8+ cells exert their effects mainly by two mechanisms, cytolytic attack on target cells or secretion of interleukins and cytokines. When CD8+ cells are stimulated to 'attack' target cells – by interaction between the TCR on the CD8+ cells and the peptide on the MHC class I molecule of the target cell – they release perforin, a molecule that produces channels in the plasma membrane of the target cell leading to lysis. In addition, CD8+ cells secrete granzymes (serine esterases), which pass through the channels in the target cell and trigger apoptosis. Effector CD8+ cells are not destroyed in this process and survive to kill additional 'prey'.

CD8+ cells also release a number of cytokines, such as IFNγ, TNFα and IL-2. These cytokines can play an essential role in clearing virus-infected tissues, either by initiating apoptosis or by purging cells without cell death – a phenomenon that is not well understood. The relative role of cytolysis versus cytokines in clearing an infection depends upon the virus. Thus, CTLs play an important role in LCMV infection, while cytokines are more important in HBV infection (Figure 6.8). In addition, cytokines initiate an inflammatory response by exerting a chemotactic effect that draws monocytes and other cells to the site of infection, leading to the elimination of virus and the removal of dead cells.

CD4+ cells can also act as effector cells under certain circumstances. Mice in which β 2 microglobulin gene has been inactivated (such mice fail to produce MHC class I molecules or CD8+ cells) will nevertheless produce virus-specific CTLs with a CD4+ phenotype. In a few viral infections of humans, the CD4+ subset of T lymphocytes can be shown to mediate cytolysis and cloned CD4+ cell lines can also be shown to attack virus-specific target cells. However, CD4+ cells usually represent only a small fraction of total CTL lymphocytes and, under physiological conditions, CD8+ lymphocytes usually constitute the major population of effector cells.

It was noted above that CTLs can initiate a process of viral clearance without necessarily destroying infected cells. Thus, in the HBV model (Figure 6.8), cytokines cause a downregulation of viral mRNA transcripts and their cognate proteins. In this transgenic model, 100% of hepatocytes carry the viral genome and clearance cannot involve cell death since the mice undergo a mild disease from which they recover. A similar phenomenon has been described for clearance of LCMV from neurons by transferred virus-specific CD8+ T lymphocytes.

The ability of effector T cells to purge intracellular viral RNA and proteins in a non-cytolytic manner is similar to the effects of antibody and is a remarkable phenomenon that remains to be explained. Studies with Sindbis virus, an alphavirus, indicate that antibody and effector T cells act synergistically to facilitate viral clearance. It appears that IFNγ and perhaps other cytokines,

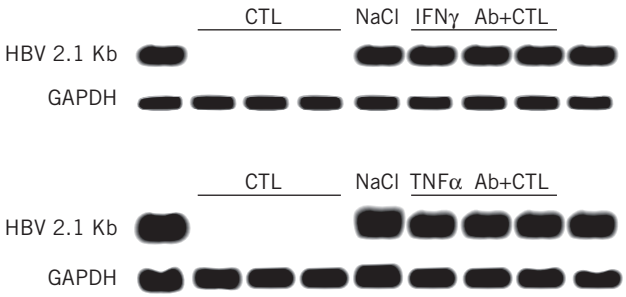

FIGURE 6.8 The CD8+ -mediated clearance of some virus infections depends upon cytokines rather than upon cytolytic activity. The figure shows the amount of RNA specific for HBV (hepatitis B virus) in the liver of transgenic mice that express the whole genome of HBV. When these mice are adoptively immunized with CD8+ cells from a non-transgenic histocompatible mouse immunized with HBV, the transferred cells clear the virus. This effect is abrogated by antibodies directed against either IFN-γ or TNF, implying that those cytokines are responsible for viral clearance. After Guidotti LG, Ando K, Hobbs MV *et al.* Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a non-cytolytic mechanism in transgenic mice. *Proceedings of the National Academy of Sciences* 1994, 91: 3764–3768, with permission.

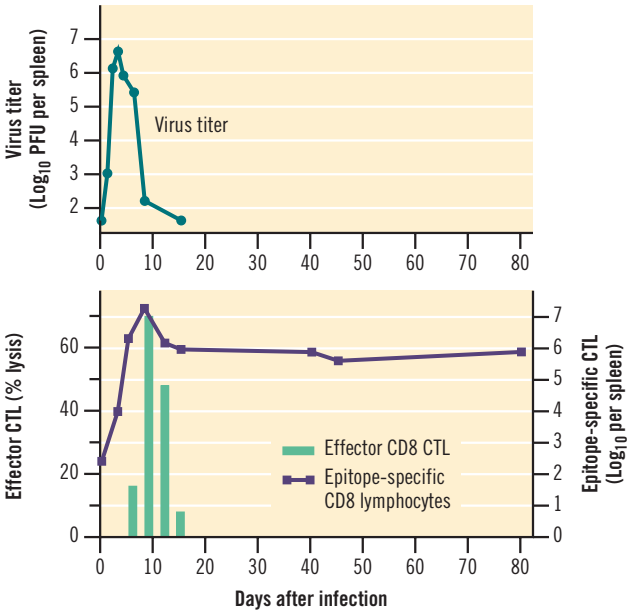

FIGURE 6.9 The kinetics of the cellular immune response showing that effector cells are transient, while memory cells persist. In this example, mice were infected with lymphocytic choriomeningitis virus (LCMV) and spleen cells were assayed for their cytolytic activity (^{51}Cr release assay) as a measure of effector CTLs. The same cell source was also assayed for the frequency of memory CD8+ lymphocytes that recognized a single immunodominant epitope (amino acids 118–126 in the nucleoprotein). After Lau LL, Jamieson BD, Somasundarma T, Ahmed R. Cytotoxic T cell memory without antigen. *Nature* 1994, 369: 648–652; Murali-Krishna K, Altman JD, Suresh M, Ahmed R. Counting antigen-specific CD8+ T cells: a reevaluation of bystander activation during viral infection. *Immunity* 1998, 8: 177–187, with permission.

are mediators of this process. IFNγ triggers several intracellular signaling pathways and the exact mechanisms of viral clearance remain to be elucidated.

Kinetics of the cellular immune response: effector and memory T cells

The following description is based on studies in which the virus is totally cleared from the host after a period of weeks to months (Figure 6.9). Virus-specific CD8+ effector CTLs appear about one week after infection, rapidly increase to a peak at 2–3 weeks after infection and then wane by 3–6 weeks to levels that are difficult to detect. The CD8+ peak often corresponds to the period when virus is being cleared by the host. At maximum, the number of virus-specific CD8+ cells can be very high, representing up to 20% of total circulating CD8+ cells. These effector T lymphocytes are constantly undergoing apoptosis and have a short half-life, probably no more than a few days, which accounts for their rapid disappearance after the peak of viral infection.

Following the acute period of infection, effector CD8+ cells drop by 10- to a 100-fold below their peak but do not completely disappear (Figure 6.9). This residual population of antigen-committed precursor CD8+ cells is usually considered to be 'memory' T cells because of their ability to proliferate rapidly if the host is reinfected. It is possible that a subset of effector CD8+ lymphocytes, expressing the IL-7 receptor α (IL-7Rα), are the predestined progenitors of the CD8+ memory pool, a question under investigation. In contrast to effector CTLs that can be difficult to detect after the clearance of infection, memory T cells probably persist for the lifetime of the animal. CD8+ memory cells have a finite lifespan (half-life of several weeks) and they proliferate slowly to maintain homeostasis.

Memory and effector T lymphocytes can be distinguished from each other by a variety of surface markers, as well as by their content of intracellular cytokines, both of which permit them to be separated in the FACS (Figure 6.10). During the conversion of CD8+ cells from effector to memory phenotype, the surface markers CD62L, CD27 and CCR7, change from low to high expression and a subset of CD8+ cells acquires the ability to mount a robust IL-2 response. Following infection, memory CD8+ cells are found predominantly in lymphoid tissues and are sometimes called central memory cells (T_{CM}). A subset of CD8+ T cells are found predominantly in peripheral tissues, mount a robust INFγ and TNFα response and are sometimes called effector memory (T_{EM}) cells. It appears that, in response to virus reinfection, T_{EM} cells generate effector cells more rapidly while T_{CM} cells will proliferate and then differentiate so that they produce a larger population of effector cells but with a greater lag time.

The role of CD4+ helper cells in the induction of CD8+ memory cells is complex. Virus-specific CD4+ cells are not required for the induction of the early CD8+ effector response (see Figure 6.2). However, if CD4+ cells are absent during immune induction, the functional qualities of CD8+ memory cells are impaired. In contrast, the maintenance of CD8+ memory cells is not dependent upon either the presence of virus-specific CD4+ cells or the persistence of antigen.

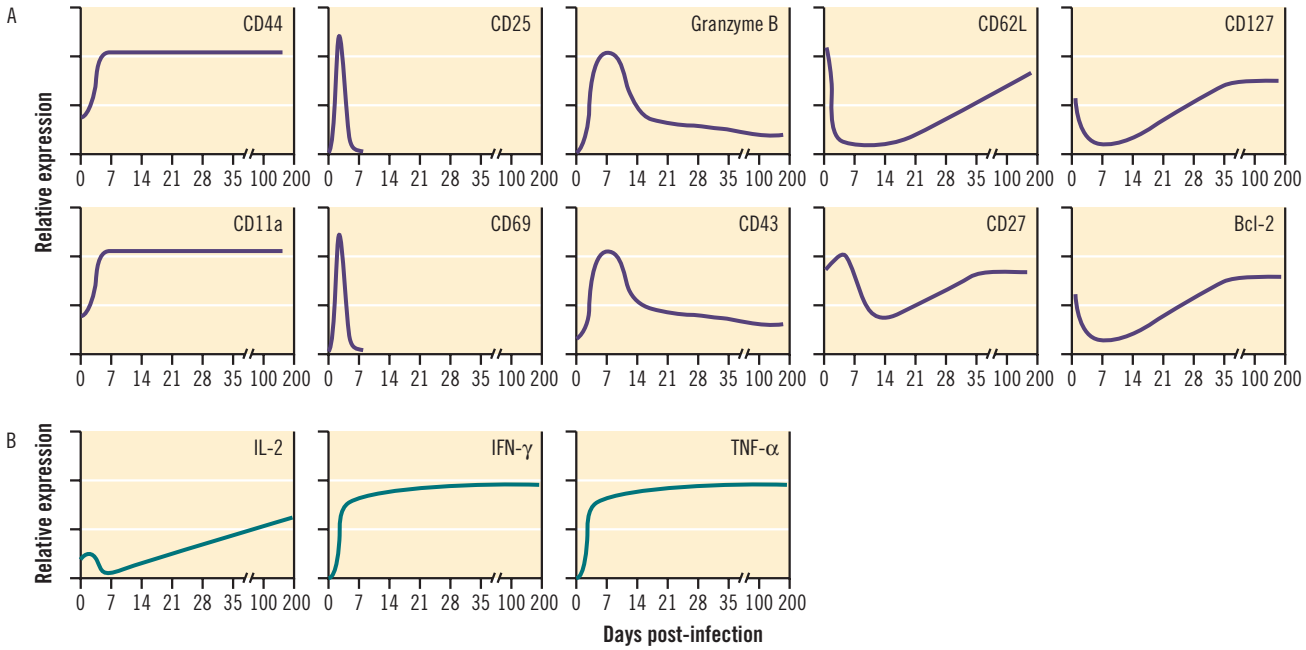

FIGURE 6.10 The conversion of CD8+ cells from effector to memory phenotype during and after acute viral infection. This graph shows CD8+ cells in the spleen of mice infected with LCMV, under conditions where the virus is cleared as in Figure 6.9. The upper panel (A) shows surface markers on CD8+ lymphocytes and the lower panel (B) shows expression of cytokines after 5 hours of in vitro antigen-specific stimulation of the same cells. Bcl-2: anti-apoptotic molecule; CD11a: adhesion molecule; CD25 part of IL-2 receptor; CD27: TNF receptor superfamily; D43: adhesion molecule; CD4+ 4: adhesion molecule; CD26L: lymph node homing receptor; CD69: signaling surface receptor; CD127: IL-7 receptor α chain. After Masopust D, Kaech SM, Wherry EJ, Ahmed R. The role of programming in memory T-cell development. *Current Opinion in Immunology* 2004, 16: 217–225, with permission.

IMMUNITY AS A HOST DEFENSE

Recovery from initial infection

A single host may be repeatedly exposed to the same virus. Recovery from the initial infection should be distinguished from recovery from reinfection since the relative role of various host defenses is probably quite different. The cellular immune response can produce a large number of effector cells in a relatively short time (see Figure 6.9), while the antibody response develops more slowly (see Figure 6.6). Also, effector CTLs have several mechanisms for destroying or purging virus infected cells, while antibody acts most effectively on free infectious virus that has not yet initiated cellular infection. For these reasons, the cellular immune response is probably the most important component of host defense against a primary infection, although antibodies play a synergistic role in clearing some primary viral infections.

West Nile infection of mice provides a good example of immune defenses against a primary viral infection (Figure 6.11). This virulent flavivirus is transmitted by subcutaneous injection and produces a transient viremia, following which virus crosses the blood–brain barrier, enters the central nervous system, infects neurons and produces acute encephalitis. In this experimental system, some mice recover and clear the virus, while others succumb. When B cells are deleted (by gene knockout), the mortality increases and the mice fail to clear the virus effectively. When CD8+ cells are deleted (another gene knockout) the mortality also rises; however, viremia is rapidly terminated, although the virus is not cleared from tissues. In this example, it appears that both limbs of the immune response are necessary for an optimal host response to initial infection, but that antibody plays a more important role in the termination of viremia while cellular immunity is critical for clearance of virus from tissues.

Protection against reinfection

There are two salient differences between the naïve host and the host that has been previously infected (or vaccinated) and has developed an immune response to a specific virus. First, the immune host usually maintains a significant titer of antiviral antibodies capable of immediately interacting with virus. Second, the immune host maintains virus-specific memory cells, both CD4+ and CD8+ lymphocytes and, on re-exposure to antigen, these cells proliferate and mature to effector cells more rapidly than during primary immune induction (see Figures 6.5, 6.6, 6.9).

Antibodies circulate in the plasma and will also be present in mucosal fluids, the antibody class and concentration depending on the prior immunizing experience. These pre-existing antibodies can play a significant role in protecting the immune host against the virus. Classical experiments that demonstrate the efficacy of antibodies have been conducted for many viruses using a passive protection protocol. Experimental animals are pretreated with virus-specific immune serum and are then challenged with virus, in comparison with appropriate controls (Figure 6.12). Even a modest level of neutralizing

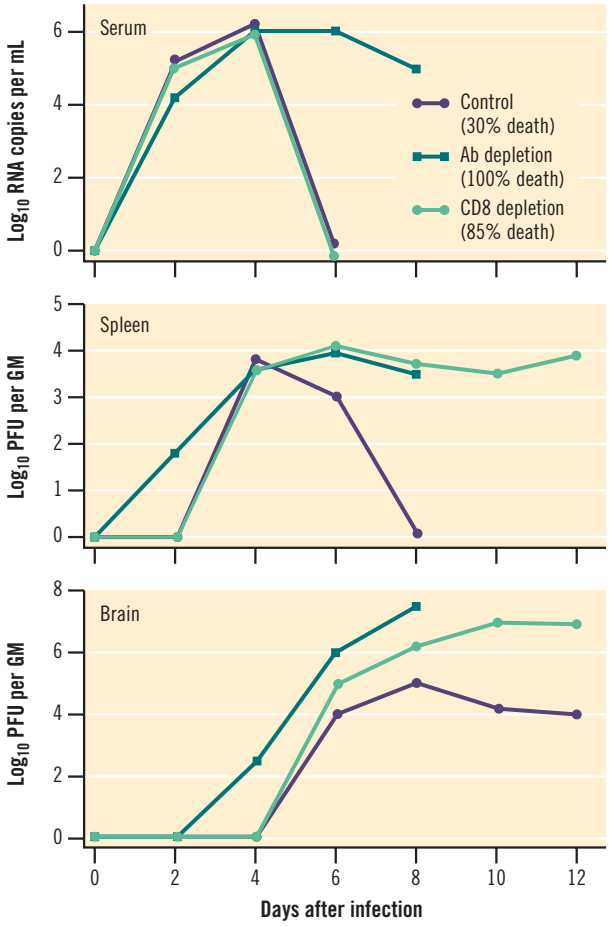

FIGURE 6.11 Both arms of the immune response can play a role in host defense against primary viral infection. West Nile virus (virulent wildtype strain), a flavivirus, was injected by the subcutaneous route in young adult mice, in which it produced a viremia followed by invasion of the central nervous system and encephalitis. Some normal animals survived and cleared the virus and were compared with congenic mice lacking CD8+ T cells (CD8+ α chain $^{-/-}$) and congenic mice that were deficient in B cells and antibody production (μ MT mice). Abrogation of either antibody or cellular immune defenses reduced host survival and viral clearance, but the patterns were different in some respects. After Diamond MS, Shrestha B, Marri *et al.* B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. *Journal of Virology* 2003, 77: 2578–2586; Shrestha B, Diamond MS. Role of CD8+ T cells in control of West Nile virus infection. *Journal of Virology* 2004, 78: 8312–8321, with permission.

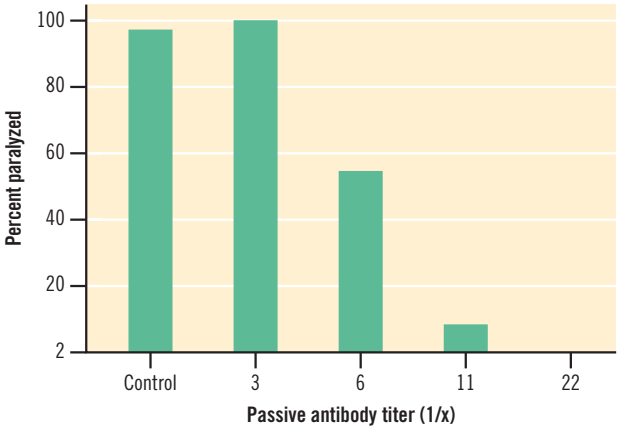

FIGURE 6.12 Passive antibody provides protection against a pathogenic virus challenge. In this instance, the challenge is the intramuscular injection of the virulent Mahoney strain of type 1 poliovirus and almost all of the control animals developed paralytic disease. A titration was conducted of different doses of anti-poliovirus antiserum, given one day prior to virus. The results indicate that protection is dose dependent and that a titer of ~1:6 in the recipient provided 50% protection. After Nathanson N, Bodian D. Experimental poliomyelitis following intramuscular virus injection. III. The effect of passive antibody on paralysis and viremia. *Bulletin of the Johns Hopkins Hospital* 1962, 111: 198–220, with permission.

antibody may protect recipients against the disease consequences of infection. Protected animals (or humans) undergo reinfection, but the virus fails to disseminate to target organs or to replicate to sufficient titer to exceed a disease threshold. The efficacy of antibody depends upon the pathogenesis of individual virus infections and plasma antibody is particularly effective if viremia plays an essential role in the dissemination of the infection to target tissues.

Another experimental line of evidence is provided by studies in which immune animals are depleted of either B cells or CD8+ T cells, to compare the protective effects of humoral and cellular immunity. Such studies indicate that antibodies play a more important role than CD8+ cells in immune protection against virus challenge (Table 6.4).

Not only does prior infection provide antibody that exists at the time of reinfection but the re-exposure induces an anamnestic or recall response, so that the antibody titer rises more rapidly than after first exposure to the virus and to higher peak titers. Furthermore, restimulation leads to the 'maturation' of the antibody response, due to a selection of B cells that synthesize antibodies with increased affinity for the stimulating antibody. Such antibodies have greater biological activity because of their enhanced ability to bind viral antigens. The anamnestic response is readily demonstrated in studies of vaccines where the timing and amount of antigen exposure can be precisely controlled (Figure 6.13).

The cellular response to virus is also more rapid and greater upon reinfection than during primary immune induction. However, in most infections where the immune host has pre-existing neutralizing antibody and where such antibody can offer significant protection by itself, the cellular response is probably less crucial than antibody.

Mucosal immune responses

Most viruses infect via a mucosal portal of entry, so that mucosal immunity is potentially important, particularly in protection against reinfection. Both antibody and CD8+ cells participate in the mucosal immune response, which differs in certain important ways from the systemic response.

Plasma cells are found in all the submucosal tissues, both in the connective tissue and in lymphoid tissues such as the tonsil and Peyer's patches in the intestinal wall. A high proportion of mucosal plasma cells secrete

Day of vaccination	Immune manipulation	Neutralizing antibody on day 22	Monkeypox infection	Fatal infection High titer replication monkeypox virus
Day 0	None	800–6400	Day 28	0/4
Day 0	B cell depletion Days −8, −1, 7, 15	42–59	Day 28	3/4
Day 0	CD8+ depletion Days 27, 29, 31, 42	268–2963	Day 28	0/4

TABLE 6.4 Antibody is more important than cellular immunity in protection of immune animals against virus infection. Rhesus macaques were immunized with vaccinia virus (dryvax) and then challenged 28 days later with monkeypox virus, which causes a highly lethal infection in monkeys, similar to smallpox in humans. B cells were depleted by treatment with an anti-CD20 antibody prior to and after vaccination and CD8+ T cells were depleted before and after monkeypox challenge
After Edgehill-Smith Y, Golding H, Manischewitz J *et al.* Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. *Nature Medicine* 2005, 11: 740–747, with permission.

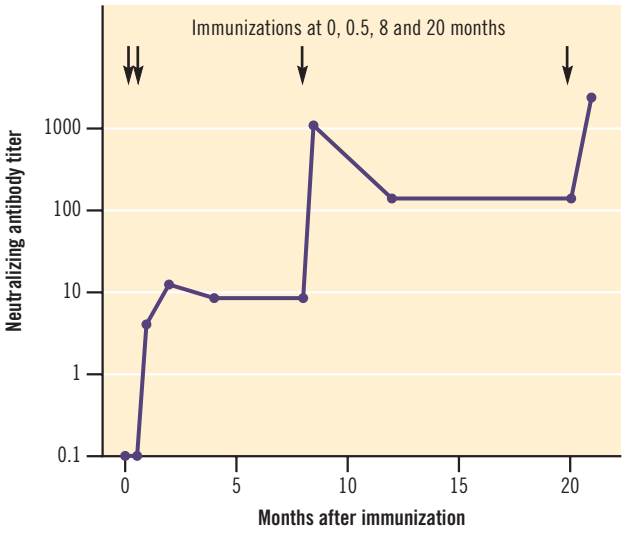

FIGURE 6.13 Re-exposure to an antigen elicits an anamnestic antibody response. Seronegative subjects were given multiple doses of an inactivated poliovirus vaccine (IPV), two priming doses, two weeks apart, followed by 'boosts' 8 and 20 months later. After Salk J. Considerations in the preparation and use of poliomyelitis virus vaccine. *Journal of the American Medical Association* 1955, 158: 1239–1248, with permission.

IgA that is assembled as a dimer bound to secretory piece, another peptide. In this form, IgA is preferentially targeted for secretion into the mucosal lumen. In addition, serum IgG escapes across the capillary endothelium and appears in mucosal tissues as a passive transudate. Both types of antibody can provide a barrier at the mucosal surface.

Mucosal IgA antibody tends to be relatively transient, lasting only months to years, in contrast to long-lasting serum antibody. This may be due to the persistence of antibody producing plasma cells in the bone marrow, which account for persistent serum antibody (see Figure 5.6) while actively secreting plasma cells do not persist in peripheral sites such as the mucosa.

Mucosal tissues contain both CD8+ and CD4+ cells capable of mounting a cellular immune response. Furthermore, mucosal T cells are distinguished from those in central lymphoid organs by their tendency to home to mucosal sites. As a result, presentation of an antigen to a mucosal surface results in a different distribution of CD8+ effector CTLs than does systemic presentation (intravenous, intraperitoneal, intramuscular, subcutaneous) of the same antigen. Furthermore, mucosal immune induction can produce greater protection than systemic immunization against a mucosal challenge with a virus (Table 6.5).

The mucosal immune system presents a biological paradox. On the one hand, the gastrointestinal tract is constantly exposed to foreign antigens that are benign in nature, such as ingested food proteins and commensal intestinal bacteria; these immunogens elicit 'tolerance', i.e. the absence of an immune response. On the other hand, the host must recognize pathogenic viruses that invade via the gastrointestinal route and raise a defensive response to repel or control these parasites. Although the dual role of the mucosal immune system has long been recognized, the underlying mechanisms are incompletely understood and are the subject of ongoing research. One hypothesis is that alternate immune responses are determined during the initial encounter with foreign antigens. This hypothesis suggests that if that the foreign antigen is presented in association with 'danger' signals or other triggers of innate immunity (such as toll-like receptors), defensive immunity may be elicited. On the other hand, it is hypothesized that some professional APCs can be programmed to initiate an anergic response, if they first encounter a foreign antigen in a chemical and cellular milieu that favors tolerance.

Escape from immune surveillance

The contest between virus and host is a never ending one, but the virus has the advantage that it can undergo more rapid evolution. Not surprisingly, viruses have developed a number of mechanisms to evade immune surveillance

		Immunization		
		Subcutaneous	Rectal	Not immunized
CTL response (% lysis)	Spleen	68%	45%	ND
	Peyer's patches	3%	35%	ND
	Lamina propria	5%	30%	ND
Rectal challenge	Virus titer log 10 per ovary	8.3	4.3	8.3

TABLE 6.5 Mucosal and systemic immunizations induce different distributions of effector T lymphocytes. Mice were immunized with a gp160 peptide of HIV either by subcutaneous or intrarectal routes. Assays showed that antigen-specific CTLs were found in the spleen regardless of route of immunization but in intestinal mucosal sites only after rectal immunization. Correspondingly, resistance to rectal challenge with a vaccinia virus incorporating the gp160 antigen was greater in animals immunized by the intrarectal route. Readout was based on the titer of vaccinia virus in the ovary 6 days after virus challenge
After Belyakov IM, Ahlers JD, Brandwein BY *et al.* The importance of local mucosal HIV-specific CD8+ cytotoxic T lymphocytes for resistance to mucosal viral transmission in mice and enhancement of resistance by local administration of IL-12. *Journal of Clinical Investigation* 1998, 102: 2072–2081.

and persist in the infected host. These include tolerance, in which viruses are treated as self antigens, latency in which the viral genome persists in the absence of gene products and therefore cannot be recognized by the immune system and other still more byzantine strategies. Viral persistence is discussed in Chapter 10.

REPRISE

Viral infection induces an antigen-specific immune response that has two main effector components, antibodies and effector lymphocytes. Immune induction involves cooperative interaction of several cell types, professional APCs – antigen presenting cells – (dendritic cells, macrophages and B lymphocytes), helper cells (CD4+ T lymphocytes) and effector cells (plasma cells, CD8+ T lymphocytes and NK cells). Induction also involves the processing of protein antigens into oligopeptides that are bound to major histocompatibility (MHC) molecules on the surface of APCs and are recognized by T cell receptors (TCRs) on CD4+ and CD8+ T lymphocytes. In contrast to T cells, B cells recognize both linear and conformational epitopes on native proteins. Following a primary virus infection, antibodies are continually produced over a long period of time (although at a submaximal level), while effector CD8+ cells disappear almost completely. On second exposure to a virus, there is a relatively rapid anamnestic response with an increase in antibody production and the reappearance of effector CD8+ T lymphocytes. During primary viral infection, the cellular immune response plays an important role in controlling and clearing the virus, while antibody is less important. Following clearance of a primary infection, the immune response provides protection against reinfection, mediated mainly by antibody while the cellular response is less significant. There are many variations on these general patterns, depending upon both the characteristics of the virus and of the infected host.

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Chapter 7

CHAPTER CONTENTS

IMMUNE MECHANISMS OF VIRUS-INDUCED DISEASE

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ANTIBODY-MEDIATED IMMUNOPATHOLOGY
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Antibody-mediated immunopathology: human disease

VIRUS-INITIATED AUTOIMMUNITY

REPRISE

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FURTHER READING

Virus-Induced Immunopathology

Neal Nathanson and Rafi Ahmed

IMMUNE MECHANISMS OF VIRUS-INDUCED DISEASE

Most virus diseases are caused by virus-cell interactions that either lead to cytolysis or cause cellular dysfunction, as described in Chapter 4. However, sometimes viral disease is mediated by the immune response to infection rather than by the infection itself. This is particularly true of viruses that are relatively non-cytopathic so that infected cells are not immediately destroyed. In such instances, the very same cellular or humoral immune responses to viral antigens that serves as host defenses, by containing and clearing the virus invader, can also mediate a pathological response. Although, at first glance this appears paradoxical, it reflects the dynamic balance between virus and host (Table 7.1). If the immune response clears the infection by destroying a small number of virus-infected cells, the host survives with minimal symptoms and no permanent damage. If a large number of cells are infected before immune induction, the same immune-mediated destruction can cause severe or fatal pathological consequences.

Every component of the immune response is capable of causing disease, although the mechanisms and manifestations will differ. Antibody–antigen complexes, cell-mediated cytolysis, or inflammation are each responsible for some instances of virus-induced immunopathology. Table 7.2 lists selected examples of immune-mediated viral diseases of animals or humans.

What is the evidence that a virus disease is immune-mediated? In some examples, such as hepatitis B virus (HBV), the first clue is that the specific virus can cause persistent infections without apparent illness, suggesting that disease is caused by an indirect mechanism. In other examples, pathological examination reveals the hallmarks of immunopathology, such as lesions containing antigen–antibody complexes, complement and inflammatory cells.

If the disease can be reproduced in an experimental animal, it is then possible to dissect the mechanism by use of manipulations, such as immuno-suppression, genetic knockouts of components of the immune system, adoptive immunization, transgenic animals expressing viral genes and the like. When mice infected with lymphocytic choriomeningitis virus (LCMV) are also treated with an immunosuppressive drug, cyclophosphamide, they fail to develop acute convulsive disease (Figure 7.1). Although the animals appear healthy, the virus replicates at a high level and produces a life-long persistent infection of the brain and lymphoid tissues. The protective

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Type of infection	Rate and magnitude of virus replication	Rate and magnitude of immune response	Tissue destruction	Outcome of infection	Role of immune response
Acute	Moderate	Robust	Restricted	Recovery	Protects
Acute	Slow	Moderate	Restricted	Recovery	Protects
Acute	Rapid	Moderate	Extensive	Severe illness	Causes disease
Acute	Moderate	Slow	Extensive	Severe illness	Causes disease
Persistent	Slow or rapid	None	None	Silent infection	Fails to eliminate virus

TABLE 7.1 Protective contrasted with pathogenic effects of the immune response to a non-cytopathic virus. In acute infections, the relative rate and magnitude of virus replication and immune induction determine the balance between the protective and the destructive effects of the immune response. If there is little or no immune response ('tolerance') to the viral antigens, a persistent infection occurs with no tissue destruction

Host species	Virus family	Virus and disease	Pathogenic immune modality	Protection from re-infection
Human	Hepadnaviridae	Hepatitis B *Hepatitis*	CD8+ cells	Antibody CD8+ cells ?
	Flaviviridae	Dengue *Hemorrhagic fever*	Immune complexes, T cells ?	Neutralizing antibody
	Paramyxoviridae	RSV Bronchiolitis	CD4+ cells	Neutralizing antibody
Mouse	Arenaviridae	LCMV *Choriomeningitis Hepatitis*	CD8+ cells	Neutralizing antibody CD8+ cells
	Picornaviridae	TMEV *Demyelination*	CD4+ cells	Neutralizing antibody
Mink	Parvoviridae	ADV *Aleutian disease (glomerulonephritis)*	Immune complexes	?
Sheep	Lentiviridae	Visna Maedi virus *Visna (Encephalitis) Maedi (Interstitial pneumonitis)*	CD8+ cells ?	Neutralizing antibody?

TABLE 7.2 A selected list of immune-mediated viral diseases of animals or humans
ADV: Aleutian disease virus; LDV: lactic dehydrogenase virus; LCMV: lymphocytic choriomeningitis virus; RSV: respiratory syncytial virus; TMEV: Theiler's murine encephalomyelitis virus.

effect of immunosuppression and the persistence of high virus titers in healthy mice, taken together, strongly suggest that acute convulsive disease is due to an antiviral immune response rather than to virus replication per se. Further evidence of an immune-mediated disease is the ability of immune (but not naïve) T lymphocytes to reproduce acute illness when transferred into immunosuppressed mice (Figure 7.1).

CELL-MEDIATED IMMUNOPATHOLOGY

Mechanisms of cell-mediated immunopathology

CD8+ cytolytic T cells (CTLs) are the most important cellular mediator of virus-induced immunopathology. As described in Chapter 6, they attack virus-infected cells in a series of steps, including:

1. contact of the CTL and its target cell
2. recognition by the T cell receptor (TCR) on the CTL of its cognate peptide epitope presented by class I major histocompatibility complex (MHC) on the target cell
3. activation of the CTL
4. delivery of a lethal attack by release of membrane-bound granules containing perforin and granzyme B
5. fusion of granules with the plasma membrane of the target cell and polymerization of perforin monomers to form a channel in the plasma membrane of the target cell
6. osmotic swelling leading to cytolysis of the target cell.

Simultaneously, release of granzyme B (a serine protease) into the cytosol of the target cell triggers an intracellular biochemical cascade that initiates apoptosis. Although CTLs express Fas ligand that could bind to Fas on the surface of the target cell, in most instances, this is probably not an important pathway for CTL attack.

In addition to the direct cytopathic attack on target cells, activated CD8+ T lymphocytes release a variety of pro-inflammatory cytokines (such as INFγ, TNFα and several interleukins) that probably play a role in some manifestations of virus-induced immunopathology. Also NK cells, as described in Chapter 6, can attack virus-infected targets in two ways, either because virus infection downregulates MHC class I expression triggering NK cell attack (elimination of

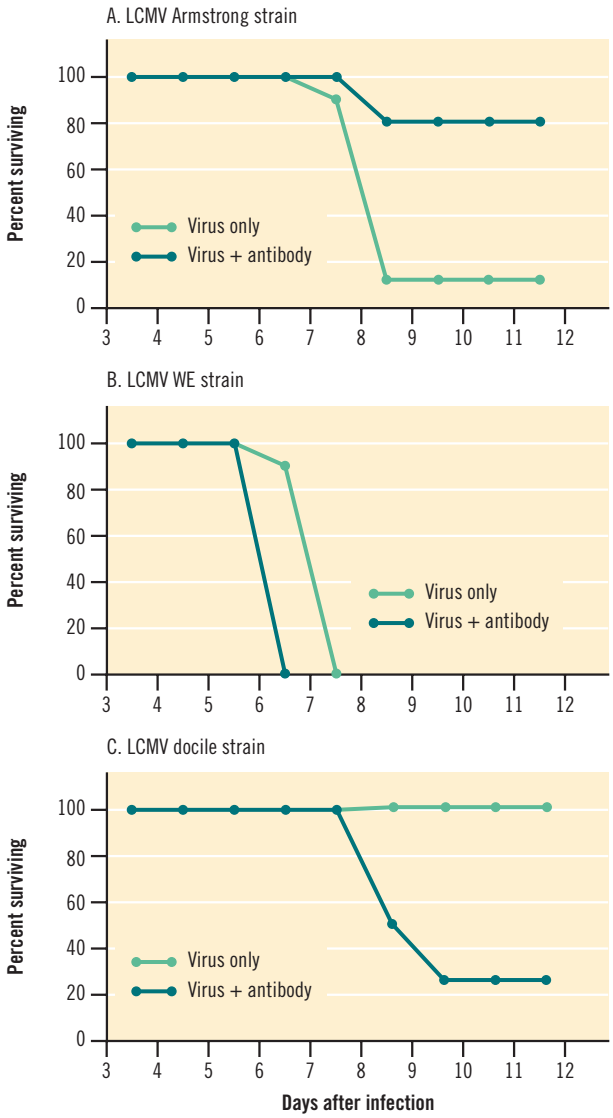


FIGURE 7.1 Experimental demonstration that a viral disease is mediated by the immune response using immunosuppression and immune reconstitution. This cartoon describes the protocol and results of intracerebral LCMV injection of adult mice that die of an acute convulsive state. Immunosuppression converts the infection into an asymptomatic persistent infection of the meninges of the brain and adoptive immunization of persistently infected mice reproduces the acute disease. Disease is produced by transfer of immune T lymphocytes (CD8 CTLs) but not immune antiserum or non-immune lymphocytes. After Gilden DH, Cole GA, Monjan AA, Nathanson N. Immunopathogenesis of acute central nervous system disease produced by lymphocytic choriomeningitis virus. *Journal of Experimental Medicine* 1972, 135: 860–889, with permission.

'non-self' cells), or via antibody-dependent cell-mediated cytotoxity (ADCC). However, it is not clear that NK cells play a significant role in the well-recognized examples of virus-induced immunopathology.

CD4 T lymphocytes may play an ancillary role in enhancing some CD8-mediated immunopathological syndromes. Thus, LCMV-specific CD4 T cells can produce some degree of disease in β2 microglobulin knockout mice that cannot express MHC class I molecules and therefore lack targets for CD8 effector T cells. Also, depletion of T lymphocyte subsets in immunologically intact mice reveals that CD4 and CD8 virus-specific T cells play different and synergistic effector roles in production of LCMV-induced immunopathological hepatitis. CD4+ T cells also appear to play a key role in some other examples of immunopathology, such as the demyelinating syndrome caused by Theiler's virus (described below).

The variable influence of the immune response upon this dynamic balance is illustrated by an experimental example in which the same antibody preparation can either protect, enhance or have no effect upon the outcome of infection (Figure 7.2). In this model, LCMV replicates in the meninges, producing a 'target' for potential immunopathological attack and also spreads to the spleen and other lymphoid tissues where it induces a CD8 response that mediates the choriomeningitis. Antibody reduced the hematogenous spread of the slowly replicating Armstrong strain of LCMV, preventing the induction of a robust CTL response and thereby protected most of the animals against the immunopathological disease. On the other hand, a 'docile' strain of LCMV replicates so rapidly in the lymphoid tissues that it induces 'high dose immune paralysis'. In this case, antibody reduced viral replication in the lymphoid tissues sufficiently to convert 'high dose paralysis' into a functional CTL response, that then produced lethal choriomeningitis.

Experimental examples of cell-mediated immunopathology

Lymphocytic choriomeningitis virus (LCMV)

LCMV is an arenavirus that naturally infects wild mice (Sidebar 7.1). Experiments with this somewhat obscure virus first led to the idea that viruses could cause immune-mediated disease (Chapter 1) and it still serves as a classical model of immunopathology. If LCMV is injected by the intracerebral route in adult mice, the animals sicken in about a week and rapidly die with neurological symptoms, the most striking of which is convulsions. The brain shows severe inflammation of its meninges (the external linings of the brain) but no involvement of the parenchyma (brain tissue) and examination with tagged antibodies shows that the virus replicates in cells in the meninges but not in the parenchyma. Although the pathophysiology of the convulsive state is not completely understood, it is probably mediated both by direct cytopathic attack on infected choroidal cells and by molecules released by inflammatory cells. Macrophages in the inflammatory exudate express high levels of iNOS (inducible nitric oxide synthase) and the elevated levels of nitric oxide could play a role in inducing convulsions.

As mentioned above, the LCMV model can be used to demonstrate that this convulsive disease is immunologically mediated. Syngeneic normal mice are 'immunized' by intraperitoneal injection with LCMV, which produces a transient infection leading to clearance of the virus and development of a cellular immune response. These 'immune' mice are then used as donors for either antiserum or lymphocytes. When immune lymphocytes are transferred into immunosuppressed mice that are undergoing an asymptomatic brain infection, the acute convulsive disease is reproduced (see Figure 7.1). Subsequent studies indicated that CD8 lymphocytes mediate choriomeningitis and that these very same cells also mediate clearance of the virus in intraperitoneally infected

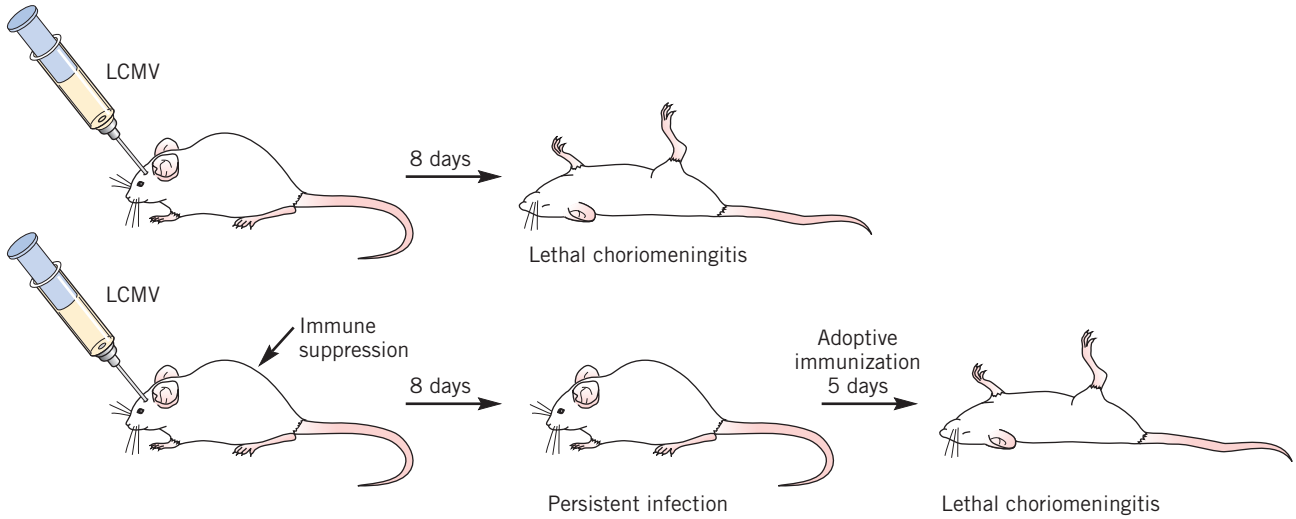


FIGURE 7.2 Antibody can have different effects on the outcome of a viral infection depending on the conditions. In this experiment, mice were pretreated with a single preparation of anti-LCMV neutralizing antibody 4 hours prior to intracerebral challenge with three different strains of LCMV that produced acutely fatal choriomeningitis in untreated animals. In this model, the virus replicates in the meninges, producing a 'target' for potential immunopathological attack and also spreads to the spleen and other lymphoid tissues where it induces a CD8 response that mediates the choriomeningitis. Panel A: antibody reduced the hematogenous spread of slowly replicating Armstrong strain of LCMV, preventing the induction of a robust CTL response and thereby protected most of the animals against the immunopathological disease. Panel B: antibody did not sufficiently slow the hematogenous spread of intermediate-replicating WE strain of LCMV and had little effect on the disease. Panel C: antibody reduced replication of the 'docile'strain of LCMV in the lymphoid tissues sufficiently to convert 'high dose paralysis' into a functional CTL response, that then produced lethal choriomeningitis. After Battegay M, Kyburz D, Hengartner H, Zinkernagel RM. Enhancement of disease by neuralizing antiviral antibodies in the absence of primed antiviral cytotoxic T cells. *European Journal of Immunology* 1993, 23: 3236–3241, with permission.

SIDEBAR 7.1

Lymphocytic choriomeningitis virus (LCMV)

Lymphocytic choriomeningitis virus is a zoonotic infection of house mice that can be transmitted to humans, probably via aerosolization of infected urine excreted by persistently infected mice. Although not an important human pathogen, LCMV has played a major role as a model in elucidating important principles in viral and general immunobiology, research that was recognized in two Nobel prizes, for the clonal selection theory of antibody production by Burnet (1960) and for the MHC restriction of cellular immune recognition, by Doherty and Zinkernagel (1996). In the 1930s, the virus was isolated by Traub from the Princeton Rockefeller Institute colony of laboratory mice and shown persistently to infect individual animals. Although colonized mice, infected at birth, did not suffer illness, when the virus was injected intracerebrally into uninfected adult mice it produced an acute lethal choriomeningitis (inflammation of the meninges or coverings of the brain). In the 1950s, Rowe showed that choriomeningitis could be prevented by X-irradiation, although irradiated mice were persistently infected, implying that disease was immunologically induced. More formal evidence of the immune nature of disease was provided in the 1970s by demonstration that disease could be prevented by chemical immunosuppression and then reconstituted by adoptive immunization (see Figure 7.2). Also, in the 1970s, Doherty and Zinkernagel used the LCMV model to demonstrate that CTLs were 'restricted' by class I MHC antigens. In the same decade, LCMV was also shown to cause several different types of immune-mediated disease, including inflammatory choriomeningitis, destructive necrosis (see Figure 7.4) and immune complex glomerulonephritis (see Figure 7.6). More recently, a number of workers have used the LCMV model to elucidate immunological memory, for both B and T lymphocytes, as well as the role of helper T lymphocytes, studies that continue to the present. Recent identification of alpha dystroglycan as the cellular receptor for LCMV has led to insights regarding the biological differences between virus strains that are immunosuppressive and those that induce a brisk immune response with rapid clearance. Immunosuppressive strains bind tightly to the receptor, replicate well in dendritic cells, interfere with antigen presentation and immune induction and may initiate a persistent infection. Strains that bind the receptor weakly do not interfere with immune induction and are usually cleared by a brisk cellular immune response.

animals. Evidence for this view is provided by experiments in perforin knockout mice whose T lymphocytes cannot mediate a CTL 'attack'. Both choriomeningitis and viral clearance are abrogated in such mice (Figure 7.3).

In some instances, immune-mediated disease causes tissue destruction in addition to inflammation. One case is the hepatitis caused by hepatotropic strains of LCMV (Figure 7.3). Another striking example is the destruction of the cerebellum in newborn rats infected by LCMV. When newborn rats are injected intracerebrally with LCMV, they develop a non-lethal infection of the brain

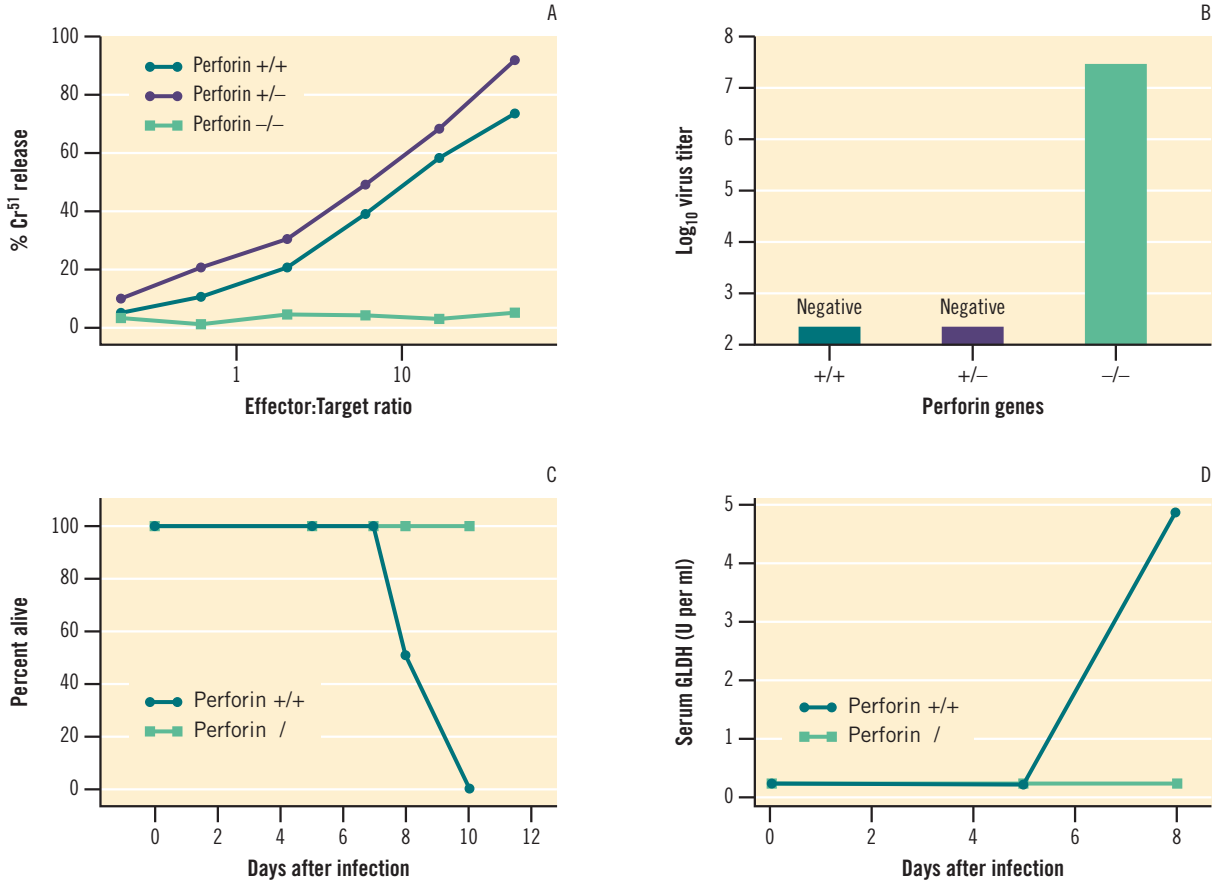


FIGURE 7.3 The use of an immunological 'knockout' to demonstrate that a virus disease is mediated by a specific component of the immune response, such as the cytopathic action of CD8+T lymphocytes. In this experimental example, mice with a normal perforin gene (+/+) are compared to mice with the gene 'knock out'(−/−) and with heterozygous mice (+/−) following infection with lymphocytic choriomeningitis virus (LCMV). The perforin gene is required for the cytolytic action of CD8 lymphocytes. Panel A: Demonstrates the absence of CD8 cytolytic activity in perforin −/− mice compared to perforin +/+ animals. Panel B: Following intravenous infection, perforin +/+mice clear the infection by day 12 after infection, while perforin −/− mice fail to clear the virus and are persistently infected. Panel C: Following intracerebral infection, perforin +/+ mice develop fatal choriomeningitis, while perforin −/− mice are infected but do not die. Panel D: After intravenous injection of a hepatotropic strain of LCMV, perforin +/+ mice develop hepatitis as reflected by a high serum level of a liver enzyme (GLDH), while perforin −/− mice are infected but do not develop liver disease. After Kagi D, Ledermann B, Burki K *et al.* Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* 1994, 369: 31–37, with permission.

concentrated in the cerebellum, a region of the brain associated with integration of locomotion. These animals survive but become severely ataxic (uncoordinated). Pathological examination indicates that the cerebellum has undergone acute necrosis and degeneration as a result of infection localized to this brain region (Figure 7.4). Immunosuppression protects against brain pathology and ataxia, even though virus replication is undiminished, again indicating an immune-mediated disease process. It is likely that this process is the direct consequence of the cytopathic action of CD8+lymphocytes.

Respiratory syncytial virus (RSV)

Some virus-induced immunopathological syndromes occur in the context of immunization. One example is respiratory syncytial virus (RSV) that produces severe, sometimes fatal, bronchopneumonia in infants. Early attempts to immunize babies employed a formalin-inactivated RSV vaccine that not only failed to protect but actually appeared to enhance pulmonary disease following natural exposure to wildtype RSV. The failure of

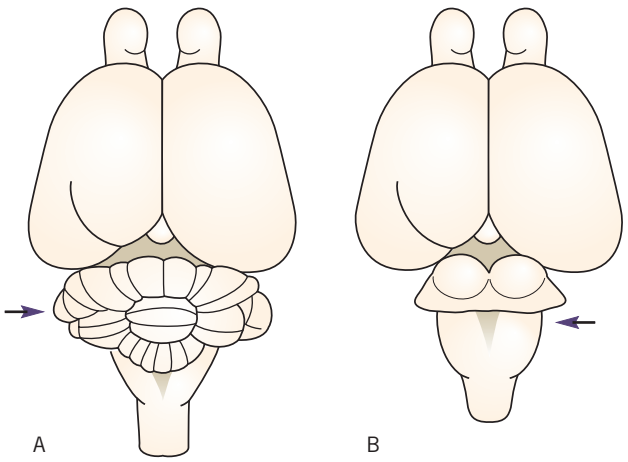


FIGURE 7.4 Immunopathological mechanisms can produce focal tissue destruction. Gross pathology of newborn rats infected with LCMV. Although both animals were infected, immunosuppression of the animal in panel A protected against the severe cerebellar involution shown in panel B. After Monjan AA, Gilden DH, Cole GA, Nathanson N. Cerebellar hypoplasia produced by lymphocytic choriomeningitis virus. *Science* 1971, 171: 194–196, with permission.

Immunization (day 0)	Depletion days 18, 0, 23	Intranasal RSV infection day 21	Alveolitis (% lung area) day 25
Formalin-inactivated RSV (All groups)	None	Yes	9.6
	CD8	Yes	3.9
	CD4	Yes	0.5
	CD8 + CD4	Yes	0.3

TABLE 7.3 Virus-induced immunopathology that is dependent upon CD4 more than upon CD8 T lymphocytes. In this protocol, mice were immunized with formalin-inactivated respiratory syncytial virus (RSV) and were subsequently infected with RSV by intranasal spray. Under these circumstances, infection produces a severe alveolitis. Mice immunized with the formalin-inactivated RSV were divided into groups and depleted of CD4, CD8 or both CD4 and CD8 cells, prior to and after challenge with intranasal live RSV. CD4 depletion (more effectively than CD8 depletion) protected against the virus-induced alveolitis. Since alveolitis could not be produced by passive transfer of antibodies alone, it was concluded that this effect is mediated, at least in part, by CD4 effector cells
After Connors M, Kulkarni AB, Firestone CY *et al.* Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. *Journal of Virology* 1992, 66: 7444–7451, with permission.

the vaccine was associated with the fact that it induced non-neutralizing antibodies that were directed against only one of the two envelope proteins of the virus, the fusion (F) protein and did not induce neutralizing antibodies against the G glycoprotein.

The lack of neutralizing antibodies explained the failure to protect but not the enhancement of disease. Subsequent depletion studies in experimental animals showed that disease enhancement was mainly mediated by CD4 cells, apparently acting as effectors rather than via their helper function for induction of antibodies (Table 7.3). Thus, disease could be induced by transfer of T lymphocytes but not by antisera from mice that were undergoing immunopathological responses. Vaccine-potentiated RSV disease in mice is associated with an effusion of pro-inflammatory T_H2 cytokines in the lung, including Il-10, Il-5 and Il-13.

By contrast, prior 'immunization' with live RSV does not trigger an inflammatory response relative to non-immunized control mice. Studies in humans indicate that natural RSV infection protects against subsequent re-infection and that protection correlates with the level of neutralizing antibody.

In contrast to immunopathology associated with formalin-inactivated RSV vaccine, the immune response (CD8+ lymphocytes) is critical for the control and clearance of infection during initial RSV infections in non-immune animals or humans. Athymic (nu/nu) mice die of progressive infection while normal controls survive.

Theiler's murine encephalomyelitis virus (TMEV)

TMEV is a picornavirus of mice that is transmitted by the enteric route and the pathogenesis of TMEV resembles that of poliovirus in primates. TMEV replicates in macrophages, produces a viremia and spreads to the central nervous system. Virulent strains of TMEV (GDVII) cause acute encephalomyelitis and death. Less virulent strains of TMEV (BeAn and DA) cause a persistent infection of the spinal cord, with the gradual development of chronic progressive spastic paralysis. This latter disease has a complex etiology, but is, at least in part , immunologically mediated.

After intracerebral inoculation of mice, less virulent strains of TMEV produce an initial infection of neurons in the gray matter of brain and spinal cord. This phase of infection is cleared by immune mechanisms about 2–3 weeks after infection. However, the virus persists in the white matter of the spinal cord, replicating primarily in macrophages at low titers and in oligodendrocytes at high titers, as measured by genome equivalents (see discussion of mechanisms of persistence in Chapter 10).

Persistent TMEV infection is characterized by demyelination (loss of myelin sheaths that surround axons) leading to spastic paralysis. Demyelination is partly due to necrosis of TMEV-infected oligodendrocytes (the cells that produce myelin). However, demyelination is mainly immune-mediated, since it can be prevented by depletion of CD4+ lymphocytes about 3 weeks after infection, at the onset of the persistent phase of infection. It is postulated that virus-specific CD4+ cells respond to viral antigens by secretion of T_H1 cytokines (Il-2 and IFNγ) and other cytokines (based on high tissue levels of mRNAs) that in turn activate macrophages. Activated macrophages secrete neutral proteases and TNFα, which attack myelin ('bystander' demyelination). Virus-induced autoimmunity may also play a role, since myelin-specific T cells are detected in TMEV-infected mice. It is presumed that the autoimmune response is triggered by myelin breakdown products that are released by the virus-induced destruction of myelin. Consistent with this reconstruction, myelin-specific T cells are first detected late in the infection, after the onset of demyelination.

Cell-mediated immunopathology: human disease

Hepatitis B virus (HBV)

Hepatitis B is one of the most important immune-mediated viral diseases of humans. Following exposure to HBV, the infection can take several different courses

and the first clues about the pathogenesis of HBV hepatitis come from a comparison of viral antigen and antibody markers in patients with diverse outcomes. Figure 7.5 compares a patient undergoing acute hepatitis and another infected subject who remains asymptomatic. The asymptomatic individual has a high titer of virus in the blood, as reflected by the amount of hepatitis B surface antigen (HBsAg) but fails to clear the infection, which often persists for many years. Such persistently infected persons exhibit little evidence of antibody (anti-HBs) against the surface antigen in their serum. In contrast, the patient with acute hepatitis clears the infection during the period when liver disease is at its peak. Shortly after clearing the virus, serum anti-HBs appears and rises to high titer. From the association of viral clearance and disease, it may be surmised that the same process that clears the virus also produces disease, another example of the dual role of the immune response (see Table 7.1).

Evidence for the immunological basis of hepatic disease and viral clearance can be derived from studies in mice that carry a transgene for the hepatitis B genome and express the major structural proteins of the virus in hepatocytes. This model has confirmed the dual roles of the immune response and has permitted a detailed dissection of the mechanisms involved (Sidebar 7.2).

If transgenic mice expressing HbsAg in their hepatocytes are injected with syngeneic CTL CD8+ lymphocyte clones directed against a specific epitope of the envelope

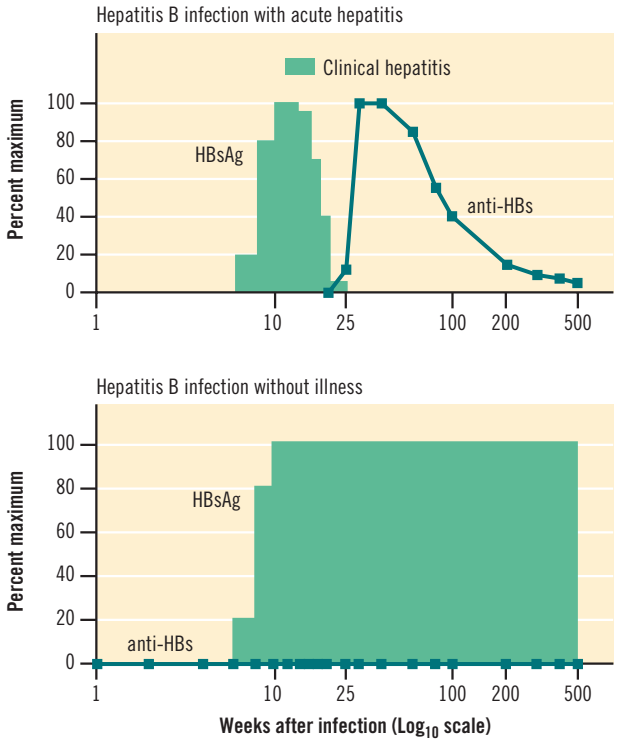


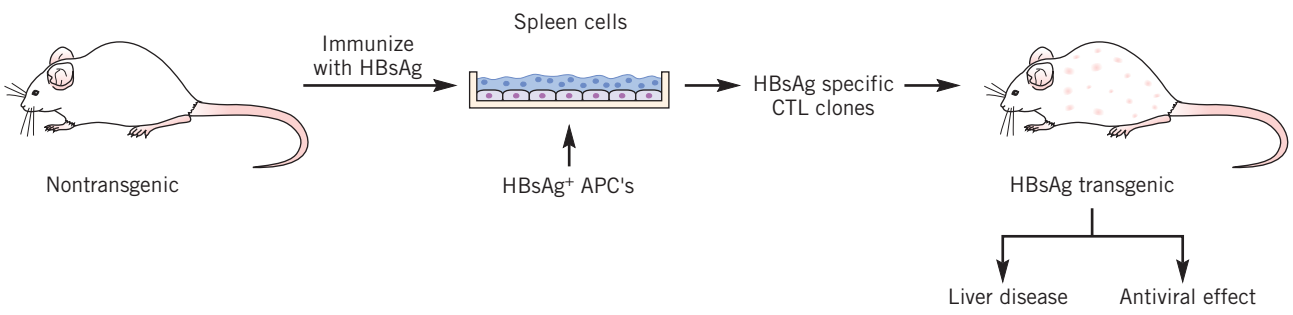
FIGURE 7.5. Hepatitis B disease likely is mediated by the immune response to infection rather than a direct effect of viral replication. Among adults, hepatitis B infection takes several different courses. Upper panel: about 20–35% of infections are accompanied by acute clinical hepatitis. Most of these patients recover completely, clear the virus and acquire lifelong immunity. Lower panel: 2–10% of individuals undergoing primary inapparent infection fail to clear their virus that persists for many years. The presence of high titers of virus in the absence of hepatitis, contrasted with the occurrence of hepatitis in patients who clear their virus, suggests that disease is not caused by infection per se, but by the immune response to the virus. Redrawn from Robinson WR. Hepatitis B virus and hepatitis D virus, in Mandell GL, Bennett JE, Dolin R (eds), *Principles and practice of infectious diseases*, Churchill Livingstone, New York, 1652–1683, with permission.

SIDEBAR 7.2

Mouse model for the study of hepatitis B virus (HBV) pathogenesis

A mouse model has been developed for the dissection of the immunological mechanisms responsible for disease production and viral clearance in HBV infection. The model focuses on a single viral protein, the envelope protein, known as HbsAg. The envelope protein occurs in three nested forms, the large, middle, and major envelope proteins, that are translated from three different RNA transcripts of the viral genome. The major and middle proteins assemble into 22 nm spherical particles and the large protein assembles into long filamentous particles about 22 nm in diameter; both particles are found at very high concentrations in the serum of HBV-infected persons.

The mouse model has two major variables. First, transgenic mice can be created that express the large, middle or major envelope proteins to elucidate the different roles of these three proteins. Second, mice can be vaccinated with immunogens that express the envelope proteins and, from the spleens of immune mice, CTL clones can be grown that are directed against individual epitopes within each of the three envelope proteins (diagrammed below). This exquisite specificity permits a more detailed analysis of mechanisms than is available for other examples of virus-induced immunopathology. Diagram based on Figure 31.5 in Chisari FV, Ferrari C. Viral hepatitis, in Nathanson N, Ahmed R, Gonzalez-Scarano F *et al.* (eds), *Viral pathogenesis*, Lippincott Raven Publishers, Philadelphia, 1997, 745–778, with permission.



protein, acute hepatitis is produced. There are two steps in the development of pathology. First, the injected CTLs attach to infected hepatocytes and induce apoptosis, but this is limited to a small number of potential target hepatocytes, probably because CTLs cannot move readily through solid tissue. Second, if the CTL clones produce large amounts of IFNγ, large numbers of white blood cells (mononuclear and polymorphonuclear cells) are attracted and produce necroinflammatory foci in which much of the cell killing is produced by non-specific 'bystander' cells distant from the CTLs. This process usually induces a limited acute hepatitis from which the mice recover, similar to most cases of human hepatitis B. If the transgenic mice express the large envelope protein, much of which is retained within the endoplasmic reticulum, then the inflammatory response progresses to a third stage, characterized by activation of liver macrophages (Kupffer cells) and progressive inflammation and necrosis that kills many of the mice; this process resembles fulminant hepatitis B that is seen in about 1% of human cases of hepatitis B.

The same process that produces hepatitis in transgenic mice also mediates viral clearance but the mechanisms are different. While hepatocellular inflammation and necrosis are produced by the direct action of CTLs, clearance seems to depend upon the cytokines, IFNγ and TNFα, that are produced in abundance by activated CTLs. Treatment with antibodies against either of these two cytokines abrogates viral clearance without altering the course of necroinflammation (see Figure 6.8). The most remarkable finding is that the cytokines downregulate all aspects of the transcription and translation of the HBV transgenes in 100% of hepatocytes without killing them and perhaps even 'cure' them of infection.

The role of anti-HBs should also be mentioned. Based on studies with passive transfer of antibody from immune subjects and on studies with HB vaccine, it appears that anti-HBs antibody is highly effective as a prophylactic to protect uninfected subjects from future exposure to HBV (discussed in Chapter 17). Antibody probably also plays a role in acute hepatitis, both in disease causation and viral clearance. When administered to transgenic mice expressing HbsAg, passive antibody causes a rapid clearance of viral antigen from the blood and a mild very transient hepatitis. This suggests that, in acute infection of humans with HBV, antibody clears viral proteins from the circulation, synergizing the CTL-mediated clearance of virus from hepatocytes. Antibody probably plays a minor role in the pathogenesis of acute hepatitis, since acute hepatic disease can be produced by individual CD8 CTL clones, in the absence of antibody, when transferred to HbsAg-expressing transgenic mice.

ANTIBODY-MEDIATED IMMUNOPATHOLOGY

Experimental examples of antibody-mediated immunopathology

Some persistent virus infections are characterized by high titers of virus or viral antigens in the blood over a long period. If any antiviral antibodies are produced during such viremic infections, antigen–antibody (immune) complexes may be formed in the circulation. The glomerular filtration system in the kidney readily passes water, electrolytes and smaller molecules, that enter the renal tubules for readsorption or excretion into the urine. Immune complexes, due to their high molecular weight, may fail to transit the glomerular filter and accumulate under the basement membrane that is situated external to the glomerular capillary wall. Immune complexes can fix complement that, in turn, will generate pro-inflammatory cytokines that draw a variety of leukocytes into the periglomerular space. The resulting chronic inflammatory process causes scarring of the glomerulus, chronic glomerulonephritis, gradual reduction of renal function and eventual kidney failure.

One example of this process is LCMV-infected mice that develop antibody-mediated glomerulonephritis and kidney failure. Mice infected with LCMV at birth have a life-long plasma viremia. However, they also produce antiviral antibodies against all of the major structural proteins of LCMV and the virus in their serum circulates as infectious immune complexes, which can be demonstrated by the fact that the infectious titer is reduced by antibodies directed against mouse immunoglobulin (Table 7.4). Both LCMV antigen and antibodies can be demonstrated in eluates of the kidneys from long-term infected mice that have massive deposits of immune complexes in their kidneys (Figure 7.6).

Long-term high titer viremias are seen in a number of persistent virus infections (see Chapter 10) and it is likely that most of them are characterized by circulating immune complexes. Depending upon the levels of antigen and antibody, such infections may lead to immune complex deposition and kidney damage (see Table 7.2). In addition to kidney disease, circulating immune complexes may produce other types of lesions, such as vasculitis.

Antibody-mediated immunopathology: human disease

Dengue

Dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) is an important example of antibody-mediated viral disease in humans. Dengue viruses are members

Treatment of sera from mice persistently infected with LCMV	LCMV titer (log_{10} LD50 per 0.02 ml)
Anti-mouse immunoglobulin	<1.0
Controls	
Normal rabbit serum	3.7
Anti-mouse albumin	3.5

TABLE 7.4 In persistent infections, viremia may exist as infectious immune complexes. In the example shown, sera from 3-month-old mice infected with LCMV at birth were treated with antibodies against mouse immunoglobulin or control antibodies and were then assayed for infectivity. The inactivation by anti-immunoglobulin antibodies is evidence that the infectious virus is bound to mouse antiviral antibodies

After Oldstone MBA. Dixon FJ. Pathogenesis of chronic of antibody production to disease in neonatally infected mice. *Journal of Experimental Medicine* 1969, 129: 483–499, with permission.

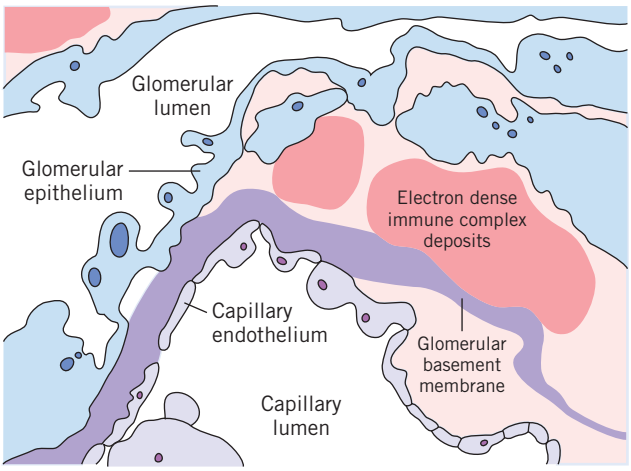


FIGURE 7.6 Immune complex deposits in the glomerulus of a mouse with chronic disease associated with persistent LCMV infection. The amorphous deposits are located between the epithelial cells of the glomerulus (that line the glomerular filtration space) and the basement membrane that underlies the capillary endothelium. Normally, a filtrate of blood passes across the endothelium and the basement membrane and then crosses the epithelial lining to enter the glomerular filtration space. Immune complexes are so large that they may become lodged in the interstitial space under the basement membrane where they gradually accumulate, leading to inflammation, scarring and loss of functional capacity of the glomerulus and chronic glomerulonephritis that is eventually fatal. After Buchmeier MJ, Welsh RM, Dutko FJ, Oldstone MBA. The virology and immunobiology of lymphocytic choriomeningitis virus infection. *Advances in Immunology* 1980, 30: 275–331, with permission.

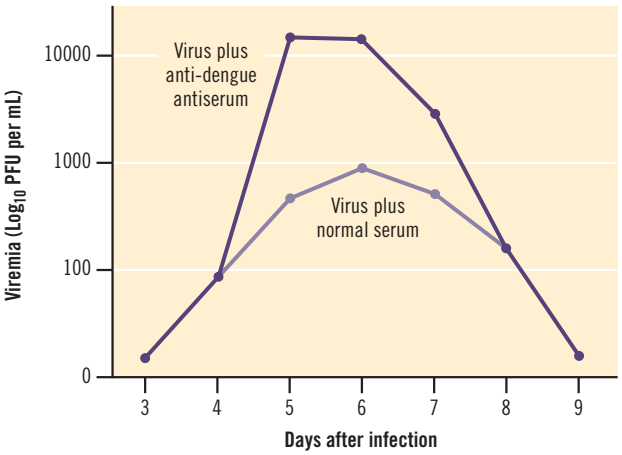


FIGURE 7.7 The replication of dengue virus is enhanced by antibody. A pair of monkeys were treated with either human antiserum against dengue virus or a control serum and 15 minutes later were infected by a subcutaneous dose of dengue virus type 2. The subsequent course of viremia shows that the antibody enhanced the infection. Similar results were obtained in four additional experiments. After Halstead SB. In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. *Journal of Infectious Diseases* 1979, 140: 527–533, with permission.

of the flaviviridae and can be grouped into four antigenically distinct types (1–4) based on serological typing. Dengue is transmitted by *Aedes aegypti* mosquitoes and is confined to certain tropical areas where the vector is indigenous. From time to time, depending upon prevalence of the vector, massive outbreaks of dengue occur in southeast Asia or in the Caribbean.

During epidemics, most infections cause an acute but self-limited febrile illness with complete recovery, but a small proportion of patients develop shock and/or a hemorrhagic diathesis, with a mortality up to 10%. Epidemiological studies indicate that DHF/DSS occurs almost exclusively in children who are undergoing a second infection with a dengue virus of a serotype different from their primary dengue infection. Also, DHF/DSS is seen in infants undergoing primary infection but who carry maternal anti-dengue antibody acquired from their mothers. These observations suggested that DHF/DSS might be mediated by an immunological mechanism. Furthermore, clinical studies have indicated that patients with DHF/DSS have both increased capillary permeability and fragility and a reduction in thrombocytes, a subcellular element required for normal blood clotting.

Cell culture studies showed that dengue viruses replicated in blood monocytes and that infection of these cells could be enhanced in the presence of small amounts of antibodies against dengue viruses. Enhancement was mediated by binding of the Fc portion of the antibody molecule to Fc receptors on the surface of monocytes, which facilitated entry of virus into their host cells. Enhancement could also be shown in monkeys infected with dengue virus and pretreated with antibodies against dengue viruses (Figure 7.7).

Although antibody-dependent enhancement may explain enhanced viral replication in secondary dengue infections, the pathophysiology of DSS is still a matter for tentative reconstruction. Monocytes or macrophages infected with dengue virus, particularly in the presence of enhancing antibody, secrete a number of vasoactive cytokines, including TNFα , IFNγ and IL-2. Also, in secondary dengue infections, CD4 and CD8 T lymphocytes specific for dengue virus antigens are activated and secrete a similar array of vasoactive cytokines. This cytokine 'storm' could account for increased capillary fragility (with associated hemorrhages) and permeability (with associated shock). In addition, circulating immune complexes (dengue viral antigens and antibodies) initiate the complement cascade, evidenced by subnormal levels of several serum complement proteins. Activated complement proteins, particularly C3a and C5a, also enhance vascular permeability and are associated with sequestration of thrombocytes.

VIRUS-INITIATED AUTOIMMUNITY

It has long been suspected that, on occasion, a virus infection might induce an autoimmune response, i.e. an immune response to antigenic determinants in a host protein. The following sequence of events could mediate such a response:

1. a virus infection elicits an immune response to a number of B and T cell epitopes on the virus-encoded proteins
2. a few of these epitopes are also shared with one or more host proteins

3. the virus-induced epitope-specific antibodies or T lymphocytes are capable of reacting with the cognate epitopes on the host proteins
4. these anti-host immune responses may elicit an autoimmune disease process.

Is there evidence of epitopes that are shared between virus proteins and host proteins? Sharing of epitopes, sometimes called 'molecular mimicry', would be a rare phenomenon (<1:10 000 000) based on chance alone. Nevertheless, there are examples of similar sequences of amino acids in viral and host proteins, as shown in Table 7.5. When antiviral monoclonal antibodies are prepared from mice shortly after infection with a variety of RNA and DNA viruses, a small number of these antibodies can be shown to react with one or more tissues in uninfected mice. In most of these instances, the shared epitope remains to be defined.

These observations suggest that viral infections may, on occasion, induce immune responses that are directed against an epitope present on both a viral and a host protein. However, it is important to recognize that an epitope-specific immune response against a host protein will not necessarily induce disease. Autoimmune disease induction probably requires that the immune response directly interferes with an essential activity of a host protein, or that the response triggers a cascade of immune responses that leads to inflammation, cell destruction and loss of function of a tissue or organ.

Myelin basic protein (MBP) can be used to illustrate this point. MBP is a major component of the myelin sheath of neuronal processes in the central and peripheral nervous system and its loss drastically curtails the conductive ability of neuronal axons and dendrites, producing dysfunction that can be progressive and fatal. If rodents are immunized with the complete protein, a severe disease (experimental allergic encephalitis, or EAE) is induced. Immunization with different segments of MBP induces immune responses against the corresponding epitopes. However, the ability to induce EAE is confined mainly to one 10-amino acid sequence (the 'encephalitogenic site') of MBP and immunization with other domains of the protein, although they elicit an immune response, does not induce EAE.

Certain viral proteins, such as the polymerase of hepatitis B virus, contain sequences similar to the encephalitogenic epitope of MBP. When a peptide based on the viral sequence is used to immunize rabbits, it induces an antibody and cellular response to rabbit MBP, as well as perivascular inflammation confined to the central nervous system. This is an experimental example of an antiviral immune response that is capable of inducing an autoimmune disease process. Although it seems plausible that virus infections trigger some cases of autoimmune disease in humans, it has been difficult to develop definitive evidence for specific diseases.

REPRISE

Although the immune response usually plays a protective role in viral infection, a few viral diseases are immune-mediated. The same immune responses that can clear viral infections can also cause immunopathology and this apparent paradox is explained by a kinetic view of infection as a race between the invading virus and host immune defenses. If the immune response is sufficiently brisk, it will clear the infection with minimal cellular damage, but if the infection is widespread when immune effector cells appear, then the attack on infected cells can produce severe disease. Immunopathology is most often seen with viruses that are relatively non-cytopathic so that infected cells are not immediately destroyed. Each major effector arm of the antiviral immune response may, on occasion, mediate disease. CD8 cytolytic T lymphocytes cause several distinct pathological syndromes in mice infected with arenaviruses, such as LCMV, including acutely fatal choriomeningitis, hepatitis and cerebellar destruction. Likewise, effector T cells are the major cause of hepatitis produced by hepatitis B virus and perhaps by hepatitis C virus in humans. In some persistent viral infections of animals, antibody binds to viral proteins to produce immune complexes that can cause progressive glomerulonephritis and vasculitis. Similar immune complex disease probably occurs in some persistent viral infections of humans, such as those caused by human immunodeficiency virus and hepatitis B virus. Antibodies probably play an important role in at least one acute human viral disease, dengue hemorrhagic fever, although the pathogenesis is not completely understood. In summary, immune-mediated viral disease is a reflection of the delicate balance between the defensive and destructive effects of the immune response and of the race between viral invaders and the defenses mounted by the infected host.

Protein	Sequence	Immunological cross-reactivity demonstrated
Poliovirus VP2	*STTKESRGTT*	Yes
Acetylcholine receptor	*TVIKESRGTK*	
Papilloma virus E2	*SLHLESLKDS*	Yes
Insulin receptor	*VYGLESLKDL*	
Rabiesvirus glycoprotein	*TKESLVIIS*	Yes
Insulin receptor	*NKESLVISE*	
HIV p24	*GVETTTPS*	Yes
IgG constant region	*GVETTTPS*	
Measles virus P3	*LECIRALK*	No
Corticotrophin	*LECIRACK*	

TABLE 7.5 Potential shared epitopes between viral and host proteins. The following table lists examples of sequences where there are at least 6 sequential identical amino acids. In most of these instances, immunological cross reactivity has also been demonstrated
After Oldstone MBA, Molecular mimicry and autoimmune disease. *Cell* 1987, 50: 819–820, with permission.

ACKNOWLEDGEMENT

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Chapter 8

Virus-Induced Immunosuppression

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INTRODUCTION

As a general rule, infections stimulate an immune response specific for the antigens of the virus. However, in a few instances, virus infections suppress the immune response. Immunosuppression is often 'global', i.e. it affects responses to many antigens. However, in some instances suppression is quite specific for the infecting virus, often associated with antenatal or perinatal infections. The mechanisms of immunosuppression are diverse (Table 8.1) and include:

1. replication of the virus in one of the cell types involved in immune induction, such as antigen presenting cells (particularly macrophages or dendritic cells) or CD4+ (helper) T lymphocytes, leading to the induction of cellular apoptosis or aberrant production of cytokines
2. tolerance, often associated with fetal or newborn infection, produced by clonal deletion of T lymphocytes that respond to viral antigens
3. perturbation of the immune response due to specific effects of individual viral proteins that act on infected or uninfected cells in a variety of ways.

Immunosuppression associated with a virus infection was first described about 100 years ago by von Pirquet who noted that patients lost their tuberculin sensitivity (skin test reaction to the antigens of *Mycobacterium tuberculosis*) during and after measles (Figure 8.1). With the occurrence of AIDS (acquired immunodeficiency syndrome), virus-induced immunosuppression has attracted renewed attention and detailed investigation. Animal models of immunosuppression have provided much useful information about its dynamics and mechanisms.

MECHANISMS OF IMMUNOSUPPRESSION

Viral infection of lymphocytes, monocytes and dendritic cells

A number of viruses can replicate in cells of the lymphoreticular system. If the virus destroys a cell type that is critical to immune induction, such as antigen-presenting cells or a specific class of T lymphocytes, immune induction may be impaired resulting in immunosuppression. A few salient examples will illustrate this mechanism of suppression.

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Mechanism	Virus	Host	Degree of immuno-suppression	Virus-specific or broad
Attack on one or more cells of the lymphoreticular system	HIV	Human	Marked	Broad
	LCMV	Mouse (adult)	Marked	Broad
	CDV	Dog	Marked	Broad
Fetal infection leading to tolerance	Rubella	Human	Moderate	Specific
	LCMV	Mouse	Moderate	Specific
Perturbation of cytokine homeostasis and intracellular signaling	Measles	Human	Moderate	Broad
Viral proteins acting as viroceptors or virokines	HSV	Human	Mild	Specific
	Vaccinia	Human	Mild	Specific
Suppressor T lymphocytes	FLV	Mouse	Moderate	Broad

TABLE 8.1 Some mechanisms of virus-induced immunosuppression
Abbreviations: CDV: canine distemper virus; FLV: Friend leukemia virus; HIV: human immunodeficiency virus; HSV: herpes simplex virus; LCMV: lymphocytic choriomeningitis virus.

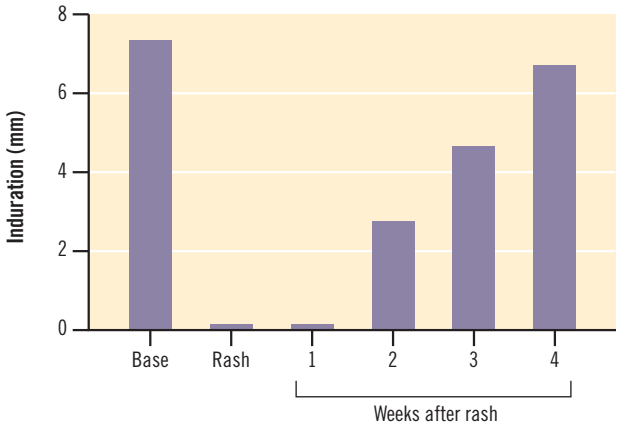

FIGURE 8.1 Immunosuppression during a viral infection. The effect of acute measles infection upon delayed-type hypersensitivity (tuberculin skin test). Immunosuppression occurs during the incubation period and the immune response exhibits maximal reduction by the time the rash has developed and gradually returns to normal over the next month. Base: tests at 1 and 2 weeks prior to measles. After Tamashiro VG, Perez HH, Griffin DE. Prospective study of the magnitude and duration of changes in tuberculin reactivity during uncomplicated and complicated measles. *Pediatric Infectious Diseases Journal* 1987, 6: 451–454, with permission.

Lymphocytic choriomeningitis virus (LCMV) and antigen-presenting dendritic cells

Certain strains of LCMV, experimentally selected, are quite immunosuppressive in adult immunocompetent mice. One immunosuppressive strain, clone 13, has been compared with a non-immunosuppressive strain (Armstrong strain) from which it was derived, to elucidate the mechanism of immunosuppression. It appears that clone 13 preferentially infects macrophages and dendritic cells, resulting in a generalized suppression of immune responses. The preferential replication of clone 13 – relative to Armstrong – in macrophages in the lung is shown in Figure 8.2. Immunosuppressive infections often lead to virus persistence and clone 13 produces a high titer persistent viremia while Armstrong strain causes an acute viremia that is cleared within 10 days (Figure 8.2).

The suppressive effect of clone 13 infection in adult mice is demonstrated in Table 8.2, which shows that the immunogenic Armstrong strain induces a CTL response while clone 13 does not. The suppressive effect of clone 13 is 'global', i.e. it compromises the immune response to other viruses as well as to LCMV. Table 8.3 shows that adult mice infected with LCMV clone 13 have markedly reduced responses to vaccinia, influenza and herpes simplex viruses relative to uninfected control mice.

It has recently been shown that virus infection of dendritic cells also plays an important role in the immunosuppressive action of measles virus (MV). In both instances, infection inhibits the development, expansion and migration of dendritic cells, interrupting antigen-presentation, with the dramatic effects described above. It has recently been suggested that this effect is mediated by a novel mechanism, in which virus-induced type 1 IFN blocks dendritic cell development via a STAT2-dependent signaling pathway (described in Chapter 5).

Lymphocytic choriomeningitis virus (LCMV) and CD4+ T cell dysregulation

In some circumstances, the immunosuppressive effect of LCMV is due in part to its ability to dysregulate T helper functions. Acute and persistent infections of mice with LCMV both induce virus-specific CD8+ effector T cells. However, these cells differ in their biological properties,

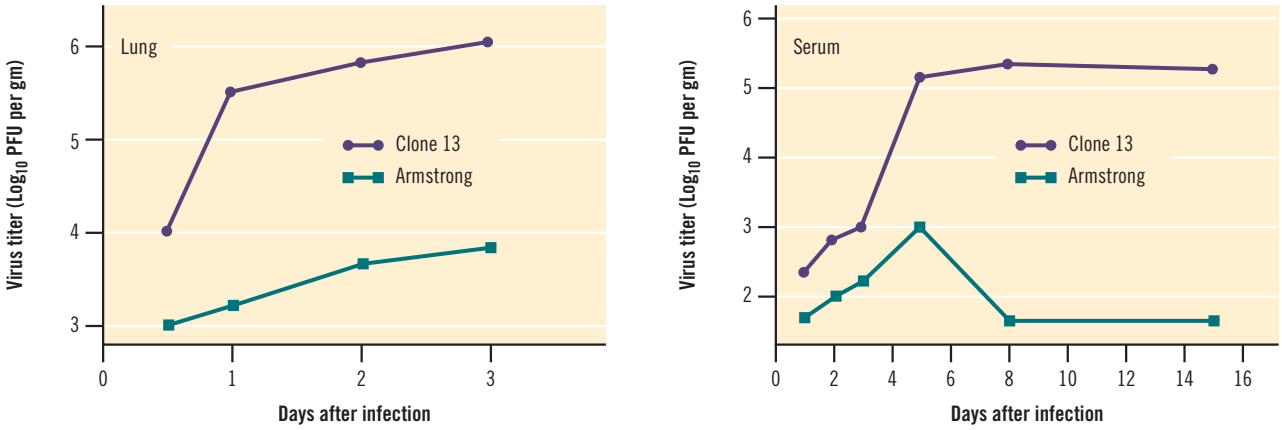

FIGURE 8.2 Comparison of the in vivo replication of an immunosuppressive variant (clone 13) and an immunogenic variant (Armstrong) of LCMV. The immunosuppressive strain replicates to a higher titer in tissues reflecting its greater ability to grow in macrophages and produces a persistent infection, while the immunogenic variant produces a short-lived viremia that is cleared by the immune response. After Matloubian M, Kolkekar SR, Somasundaram T, Ahmed R. Molecular determinants of macrophage tropism and viral persistence: importance of single amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. *Journal of Virology* 1993, 67: 7340–7349, with permission.

LCMV virus strain used to infect mice	Percent ^{51}Cr release from target cells infected with			LCMV antibody titer (log_2)
	No virus	Armstrong	Clone 13	
Armstrong (immunogenic)	0	51	45	12.7
Clone 13 (immunosuppressive)	0	7	1	12.0

TABLE 8.2 Comparison of the immune response to an immunosuppressive variant (clone 13) and an immunogenic variant (Armstrong) of LCMV. Adult mice were injected on day 0 with $10^{5.2}$ PFU of either virus and spleen cells were tested on day 8 for their ability to lyse target cells, as measured by the release of radioactive label (^{51}Cr) at an effector:target ratio of 50:1. Antibody titers in serum were measured in an ELISA assay at the same time
After Ahmed R, Salmi A, Butler LD, Chiller JM, Oldstone MBA. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. *Journal of Experimental Medicine* 1984, 160: 521–540, with permission.

Infection with clone 13 at age	CTL response (lytic units) on target cells infected with				Immunosuppression
	Herpes simplex	Influenza	Vaccinia	LCMV	
Not infected (control)	389	586	4000	16 246	None
<1 day	378	628	5769	0	LCMV-specific
6 weeks	0	56	159	0	Broad

TABLE 8.3 LCMV infection of mice as adults suppresses immune responses both to LCMV and other viruses, while infection of newborn mice suppresses responses to LCMV but not to other viruses. Mice were infected with clone 13, an immunosuppressive variant, as newborns or at 6 weeks of age and were subsequently stimulated with vaccinia, herpes simplex, influenza or the non-immunosuppressive Armstrong strain of LCMV. Their CTL responses were determined 7 days later against target cells infected with the stimulating virus. A lytic unit represents the number of T lymphocytes needed to lyse 100 target cells and the table shows the number of lytic units per spleen or lymph node
After Tishon A, Borrow P, Evans C, Oldstone MBA. Virus-induced immunosuppression. *Virology*, 1993, 195: 397–405, with permission.

because CD8+ cells from persistently infected mice lack the ability to persist in the absence of antigen, a defining characteristic of CD8+ memory cells. It is known that effector T cells can be induced by an acute viral infection in CD4 knockout mice, but that CD8+ memory T cells are not induced in the absence of CD4+ T cell help (Chapter 6). It appears that during the first few weeks after initiation of persistent infections with LCMV, the virus dysregulates CD4+ helper T cells, so that they fail to provide the functions required to induce virus-specific memory CD8+ T cells.

AIDS viruses and CD4+ T lymphocytes

HIV and related viruses (such as SIV, simian immunodeficiency virus) utilize the CD4 molecule as their primary receptor. As a result, the virus replicates in CD4+ T lymphocytes and monocytes/macrophages/dendritic cells, the major cell types that express CD4 on their plasma membranes. Monocytes undergo a relatively non-cytopathic infection with HIV but T lymphocytes, if they are proliferating, undergo a cytopathic infection. In addition, indirect mechanisms are involved in destroying CD4+ lymphocytes, as described in Chapter 14. The

Dose of LCMV (Docile strain)	Vaccinia virus infection	CTL activity, lytic units (×1000) per spleen on target cells infected with		
		LCMV	Vaccinia virus	Uninfected
Not infected (control)	Infected	ND	31	0.3
10^2 PFU	Not infected	228	ND	ND
10^7 PFU	Not Infected	<0.2	ND	<0.2
10^7 PFU	infected	<0.2	26	0.1

TABLE 8.4 Virus-induced high dose tolerance produces virus-specific immunosuppression. Adult mice were infected intravenously with a tolerogenic (Docile) strain of LCMV at two doses, a low dose (10^2 PFU) that was immunogenic and a high dose (10^7 PFU) that was tolerogenic. Some animals were also infected with vaccinia virus to test their immune responsiveness. Spleen cells were harvested 20 days after infection (immunization) and were tested for pCTLs in a limiting dilution assay (see Chapter 6 for technical details). The LCMV-tolerized mice raised a vaccinia response similar to mice that were not infected with LCMV, indicating that tolerance was virus-specific ND: not done. After Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acute infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 1993, 362: 758–761, with permission.

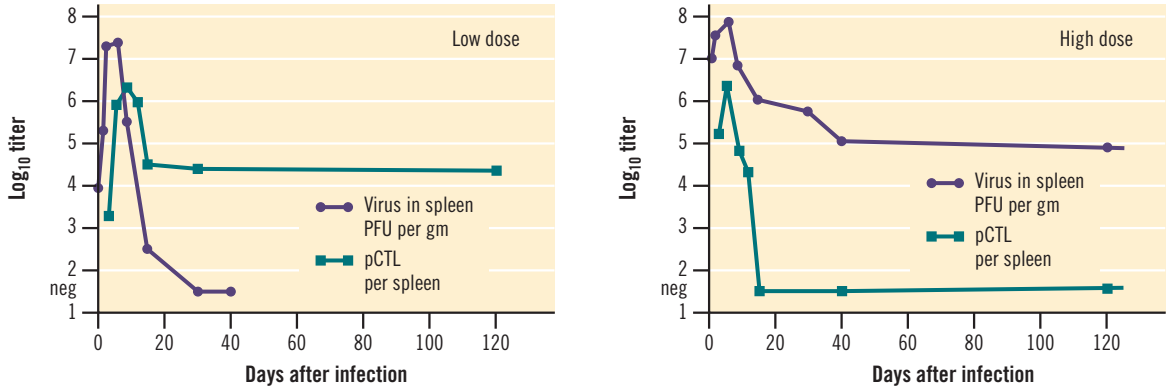

FIGURE 8.3 Virus-induced immunosuppression that resembles 'high dose' tolerance to self antigens. Adult mice were infected intravenously with a tolerogenic (Docile) strain of LCMV at two doses. The low dose (10^2 PFU) was immunogenic and induced long-lasting CTLs that cleared the infection. The high dose (10^7 PFU) induced rapid development of precursor cytolytic T cells (pCTLs) but these had totally disappeared by day 15 after infection due to the 'exhaustion' of these 'forbidden' clones, resulting in a persistent infection. After Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acute infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 1993, 362: 758–761, with permission.

depletion of CD4+ lymphocytes, that act both as T helper cells and, under some circumstances, as T effector cells, produces functional immunodeficiency that leads to opportunistic infections and the other manifestations of AIDS (see a later section of this chapter for a more extensive description).

Tolerance: virus as self

Healthy animals are immunologically 'tolerant' to their own antigens, i.e. their own proteins do not induce antibodies or cellular immune responses. There are several mechanisms for the induction of tolerance, including the thymic deletion of 'forbidden' clones of T lymphocytes, or anergy caused by the peripheral 'exhaustion' of such 'forbidden' clones. The potential for responding to a viral antigen as 'self' is readily demonstrated in transgenic mice that express a viral antigen but do not produce virus antigen-specific antibodies or T lymphocytes. Tolerogenic virus infections are often characterized by several features:

1. they are most easily induced in fetal or newborn animals
2. they are more readily induced if virus replication produces high levels of antigen
3. tolerance is virus-specific and responses to other viruses are not impaired
4. tolerance usually leads to virus persistence.

Lymphocytic choriomeningitis virus (LCMV) and tolerogenic infections

LCMV provides a well-studied model of tolerogenic infections in mice. Injected into newborn mice, LCMV usually produces a tolerogenic infection, as illustrated in Table 8.3. Such mice exhibit suppression of certain virus-specific immune responses with no evidence of LCMV-specific CTLs even though they produce LCMV antibodies that eventually cause immune complex glomerulonephritis (see Chapter 7). LCMV infects immature T cells (negative for CD4 and CD8 markers) in the thymus of neonatal mice and T cells that carry T cell receptors (TCRs) for LCMV antigens are deleted in the thymus, so that they fail to appear in the blood or peripheral lymphoid system.

Tolerogenic infections can also be induced in adult mice infected with a high dose of a tolerogenic LCMV variant. Table 8.4 and Figure 8.3 show that a low dose (10^2) of the Docile strain of LCMV induced CTL and the virus was cleared from the spleen within a few weeks.

By contrast, a high dose (10^7 PFU) of the Docile strain was tolerogenic, leading to a persistent infection. The high virus dose induced a CTL response that was even more brisk than that induced by the low virus dose, but CTL then rapidly waned and had disappeared within three weeks after infection. This phenomenon is often called 'exhaustion' and is associated with the kinetics of effector CTLs. Effector CTLs are subject to two possible fates; most of them rapidly undergo apoptosis while a minority become 'memory' cells that recycle slowly and can be stimulated to proliferate and differentiate into effector cells (see Chapter 6). Apparently, in the presence of excess antigen, all effector cells are driven into apoptosis and none become memory cells, leading to 'exhaustion' of the virus-specific CTL population. In the LCMV model, T cell exhaustion is associated with the reduced expression of Il-7Rα and of the anti-apoptotic molecule, Bcl-2.

Viral proteins that perturb the immune response

A number of viruses encode proteins that can perturb the immune response. This is particularly true of larger viruses, such as herpesviruses and poxviruses, which appear to have 'captured' cellular genes that have evolved to act as 'decoys' that interfere with antiviral host defenses. These viral proteins can interfere with the immune response through a wide variety of mechanisms and some of the best defined examples are mentioned below.

Effect on lymphocyte proliferation

In some instances, a viral protein appears to alter the ability of lymphocytes to respond to an immune stimulus. For example, many retroviruses encode an envelope protein, p15E, that reduces the proliferative response of T lymphocytes and the activation of monocytes. The effect has been mapped to a small domain within p15E and can be simulated by peptides that have the same immunodepressing sequence.

Effect on complement

After antibody has bound to its cognate antigen, the Fc portion of the antibody molecule undergoes a conformational change that results in the binding of the C1q complement protein to the Fc domain, initiating the complement cascade (see Chapter 5). The complement cascade, in turn, can lead to virolysis or cytolysis of virus-infected cells, thereby acting as a host defense. Herpes simplex virus (HSV) encodes two envelope glycoproteins, gE and gI, that can bind to the Fc moiety of the Ig (immunoglobulin) molecule. This bipolar bridging prevents the secondary effects of antibody binding, such as the activation of the complement cascade (Figure 8.4). The biological effect of the gE protein can be demonstrated by a comparison of cells infected with wildtype HSV (gE-positive) and cells infected with gI- or gE-deficient mutants, showing that the latter are relatively resistant to complement-mediated cytolysis. In addition, HSV can block the complement cascade in a second way,

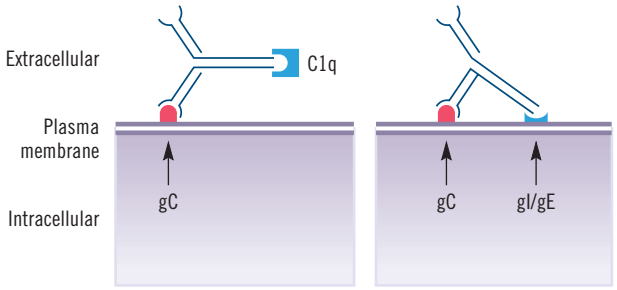

FIGURE 8.4 Cartoon to illustrate the binding of Ig (immunoglobulin) to the surface of herpes simplex virus-infected cells via gC, a viral envelope protein. Two other viral glycoproteins, gE and gI, bind the Fc domain of Ig and prevent the binding of C1q. If cells are infected with mutants defective for gE and gI, the Fc domain is available to bind C1q and initiate the complement cascade.

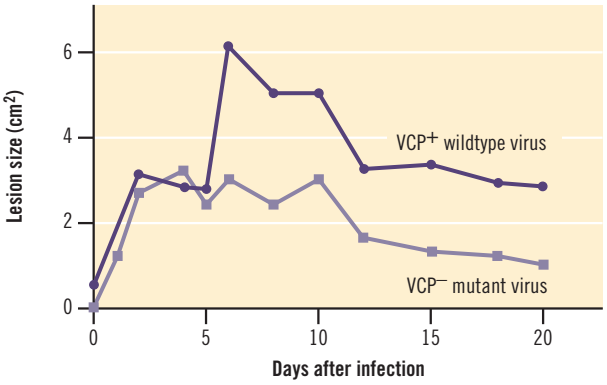

FIGURE 8.5 Viral genes can perturb the immune response by blocking the complement cascade. In this example, a comparison is made of wildtype vaccinia virus with a mutant that lacks the VCP (vaccinia complement control protein), a viral gene. Rabbits received intradermal injections of 10^6 plaque-forming units (PFUs) of the two viruses and the size of the lesions were compared (medians for three animals in each group). The lesions produced by the VCP-negative mutant were consistently smaller, beginning at 5 days after infection, suggesting that VCP interfered with the ability of the host to contain the vaccinia-induced skin lesions. After Isaacs SN, Kotwal GJ, Moss B. Vaccinia virus complement control protein prevents antibody-dependent complement-enhanced neutralization of infectivity and contributes to virulence. *Proceedings of the National Academy of Sciences* 1992, 89: 628–632, with permission.

through another one of its envelope proteins, gC, which binds C3b, one of the intermediates in the cascade.

Vaccinia virus, a poxvirus, interferes with the complement system in a different way, by blocking another element in the complement cascade. Vaccinia virus encodes VCP, a vaccinia complement control protein, that resembles a human plasma protein, C4-BP. The function of C4-BP is to monitor the complement cascade and prevent its unwanted activation, by binding to C4b, one of the intermediaries in the cascade. VCP vitiates complement-mediated immune host defenses by binding C4b and blocking the complement cascade. Its impact can be shown by comparison of wildtype vaccinia virus with VCP-minus mutants that are less pathogenic in vivo (Figure 8.5).

Downregulation of MHC class I molecules

As described in Chapter 6, CD8+ cytolytic effector cells, through their T cell receptors, recognize viral antigens

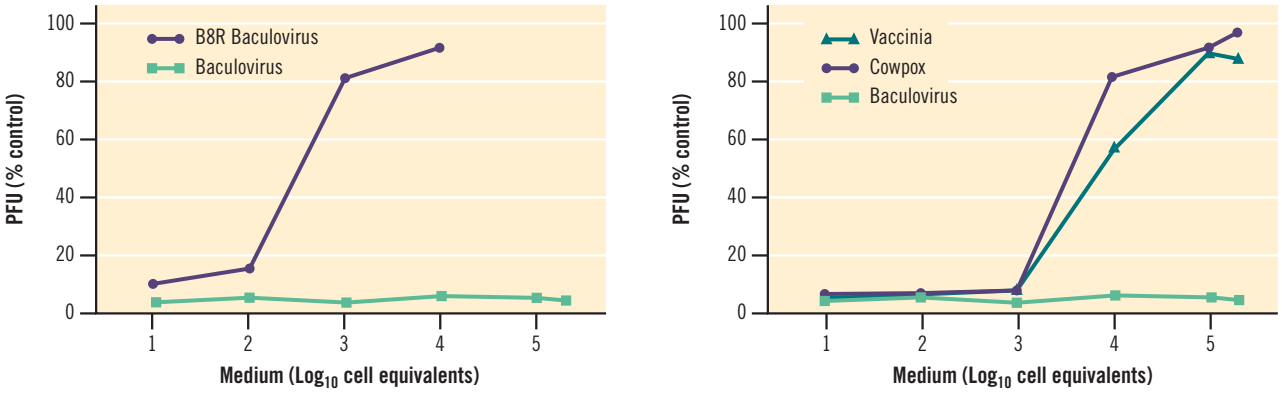

FIGURE 8.6 A viral protein that simulates the IFNγ R (interferon γ receptor) can block the antiviral action of IFNγ. The B8R protein of poxviruses is homologous to the IFNγ R and this study indicates that it can block the action of IFNγ. The figure shows an assay in which 10 units of human interferon were used to reduce the titer of Cocal, a vesiculovirus; Cocal virus and human IFNγ were present in each assay and the data are expressed relative to the control titer in the absence of IFN. Left panel: the supernates from two baculovirus cultures are compared, a control baculovirus and one that was engineered to express B8R. B8R blocks the effect of IFNγ and permits Cocal virus to replicate. Right panel: supernates from cultures infected with two poxviruses, vaccinia and cowpox, are compared with the supernates from a mock-infected control culture, to show that the media from poxvirus-infected cultures blocks the effect of human IFNγ, presumably due to the presence of the B8R protein in the virus-infected media. After Alcami A, Smith GL. Vaccinia, cowpox, and camelpox viruses encode soluble gamma interferon receptors with novel broad species specificity. *Journal of Virology* 1995, 69: 4633–4699, with permission.

presented as peptides on class I molecules, leading to lysis of virus-infected cells. Poxviruses, adenoviruses, herpesviruses and AIDS viruses encode proteins that downregulate the expression of class I molecules, which renders virus-infected cells less susceptible to cytolytic T lymphocytes.

The E1A protein of oncogenic adenoviruses reduces transcription of class I molecules. Some adenoviruses are oncogenic in animals and others are not and the oncogenic adenoviruses downregulate MHC class I expression. The non-oncogenic viruses lack this down-regulating activity; they transform cells in culture, but will not produce tumors in normal mice, although they are oncogenic in immunocompromised animals (see Chapter 4). Adenovirus also encodes another protein, E3/19K, that traps MHC class I molecules in the endoplasmic reticulum (ER) so that they never reach the cell surface. E3 is a transmembrane protein and its cytoplasmic tail encodes a small domain that retains it in the ER while its exodomain encodes a sequence that binds to Class I molecules.

Interference with cytokine functions

Poxviruses produce a number of proteins that act as 'viroceptors'. These proteins resemble and compete with cellular receptors of the host, binding the cytokine and reducing its physiological activity. Interferon γ (IFNγ) or 'immune' interferon is produced by antigen-specific T lymphocytes as part of their cytokine response to antigen (see Chapter 6). Furthermore, IFNγ can play a vital role as an immune defense, as demonstrated by treatment with monoclonal antibodies against IFNγ that can convert an innocuous into a lethal viral infection in some experimental models. Because all IFN responses are initiated by binding of IFNs to their cognate cellular receptors, proteins that mimic the IFNγR can sequester IFN and prevent its binding to the cellular receptor. Many poxviruses encode a protein (B8R) that is homologous with the cellular IFNγR and this protein binds IFN and can abrogate the protective effect of IFN in a cell culture system (Figure 8.6).

Blocking innate immunity

As understanding of the innate immune response expands, viral mechanisms that perturb the response are being uncovered. For instance, a non-structural protein of hepatitis C virus targets a cellular protein (MAVS, mitochondrial antivirus signaling protein) and thereby subverts an interferon induction pathway.

ANIMAL VIRUSES ASSOCIATED WITH IMMUNOSUPPRESSION

A number of animal viruses – from many different virus families – can cause immunosuppression of varying degrees of severity and specificity (Table 8.5). Several of these, that have served as important models to elucidate the mechanisms of suppression, are described below.

Lymphocytic choriomeningitis virus (LCMV)

LCMV is an arenavirus that occurs in nature as an indigenous virus of wild mice and has served as an important experimental model to study interactions between the immune system and viral infection (see Chapter 6). As described earlier in this chapter, some strains of LCMV are immunosuppressive under certain experimental conditions and a study of the model elucidates some of the variables that are determinants of immunosuppression.

When adult mice are infected intravenously with LCMV, the outcome depends upon the strain of virus used. Table 8.2 illustrates that the Armstrong strain of LCMV induces a virus-specific cellular immune response (CTLs), but clone 13 is immunosuppressive and fails to

Virus family *Virus group*	Virus and *disease*	Cells infected (lymphoreticular)	Manifestation of immunosuppression
Human viruses			
Paramyxoviridae *Morbilliviruses*	Measles *Measles*	Monocytes Thymic epithelial cells	Reduced DTH Enhanced infections
Togaviridae *Rubiviruses*	Rubella *Rubella*	Lymphoid cells	Persistent rubella infection
Retroviridae *Lentiviruses*	HIV *AIDS*	CD4+ T lymphocytes Monocytes	Opportunistic infections Enhanced neoplasia
Animal viruses			
Arenaviridae	LCMV *Choriomeningitis*	Dendritic cells, monocytes T lymphocytes	Persistent LCMV infection
Paramyxoviridae *Morbilliviruses*	CDV *Canine distemper*	Monocytes Lymphocytes	Encephalitis Bacterial superinfections
	RV *Rinderpest*	Monocytes Lymphocytes	Lethal gastroenteritis
Retroviridae *Lentiviruses*	SIV *AIDS*	CD4+ T lymphocytes Monocytes	Opportunistic infections
Retroviridae *Oncoviruses*	MuLV (defective variant) *MAIDS*	B lymphocytes	B, T cell dysfunctions Opportunistic infections
Parvoviridae *Autonomous parvoviruses*	FPV *Gastroenteritis* *Cerebellar ataxia*	Replicating immunocytes	Profound acute leukopenia

TABLE 8.5 Selected examples of human and animal viruses that cause immunosuppression
AIDS: acquired immunodeficiency syndrome; CDV: canine distemper virus; DTH: delayed-type hypersensitivity; FPV: feline panleukopenia virus; HIV: human immunodeficiency virus; LCMV: lymphocytic choriomeningitis virus; MAIDS: murine AIDS; MuLV: murine leukemia virus; RV: rinderpest virus.

induce CTLs, although both viruses induce similar antibody responses. Associated with immunosuppression, clone 13 induces a persistent infection in contrast to the immunogenic Armstrong strain that is rapidly cleared (see Figure 8.2). In this model, immunosuppression appears to be due to the enhanced ability of the immunosuppressive clone 13 variant to replicate in dendritic cells and macrophages (see Figure 8.2), which are destroyed by virus-specific CTLs reducing the efficiency of antigen presentation.

Morbilliviruses: canine distemper virus (CDV)

Canine distemper virus produces an acute febrile and highly fatal disease in dogs and a number of other species of carnivores. Death is due to severe encephalitis, pneumonitis, gastroenteritis and bacterial superinfections, with hemorrhage and dehydration. A similar virus, rinderpest virus (RV), infects cattle and can cause devastating epidemics with very high mortality. CDV and RV are both morbilliviruses, closely related to measles virus.

CDV infects lymphocytes and monocytes and produces a severe leukopenia, i.e. a reduction in the number of lymphocytes (both B and T cells) in the circulation and lymphoid tissues. Functionally, there is a loss of the lymphocyte proliferative response to mitogens and pre-existing responses to specific antigens also decrease or disappear. The outcome of infection is variable and dogs that recover develop prompt antibody and CTL responses to CDV, while those that die exhibit reduced or absent immune responses to the virus (Figure 8.7). The acute suppression of immune responses plays an important role in the high mortality, reducing the ability of the host to contain CDV and also potentiating superinfections with bacteria and other microbial agents. In animals that survive CDV infection, the immune responses generally recover in 1–3 months.

Retroviruses: murine leukemia virus and murine AIDS (MAIDS)

MAIDS is the name given to a disease produced in mice by a variant of murine leukemia virus (MuLV). Although the disease is called murine 'AIDS', it is produced by a replication incompetent variant of an oncogenic retrovirus and its pathogenesis is completely different from human and simian AIDS, which are caused by replication competent lentiviruses. The most prominent manifestations of MAIDS are splenomegaly and lymphadenopathy with increased susceptibility to experimental infection with many microbial agents, such as ectromelia virus, *Mycobacterium avium* and *Trypanosoma cruzi*.

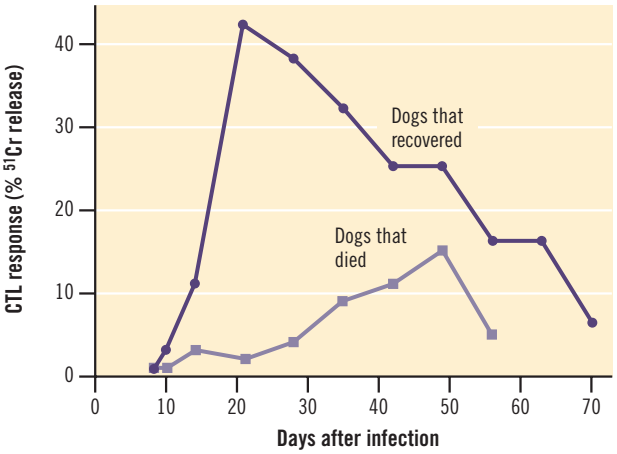

FIGURE 8.7 Virus-induced immunosuppression is associated with enhanced disease. The outcome of canine infection with canine distemper virus (CDV) is variable and those animals that undergo a fulminant and fatal course usually show marked depression of their CTL response to CDV compared to dogs that recover. This figure compares six dogs (mean values) that developed a fatal or persistent severe infection with CDV to six dogs that recovered from infection with the same virus (Cornell A75/17 strain) using a CTL assay with autologous target cells. After Appel MJG, Shek WR, Summers BA. Lymphocyte-mediated immune toxicity in dogs infected with virulent canine distemper virus. *Infection and Immunity* 1982, 37: 592–600, with permission.

The MAIDS virus is a defective variant of MuLV that was isolated in the course of experimental manipulations of laboratory-passaged retroviruses. The MAIDS virus genome lacks most of the retrovirus structural proteins and can only replicate in the presence of a non-oncogenic replication-competent murine retrovirus, which acts as a 'helper' virus by supplying the missing proteins in trans. Cells that are co-infected with the MAIDS virus and a helper virus will produce infectious virions (virus particles) that are formed of proteins encoded by the helper virus encapsidating the genome of the MAIDS virus. Such virus particles can infect target cells but, with the helper virus absent, cannot produce infectious progeny virus.

B lymphocytes infected with MAIDS virus proliferate and disseminate widely into both lymphoid and non-lymphoid tissues. Although not the primary target of the MAIDS virus, CD4+ T lymphocytes also proliferate excessively (perhaps due to cytokine dysregulation) and play an essential role in disease production. The B and T lymphocytes that undergo excessive proliferation are not able to respond to neoantigens and may displace normal lymphoid cell populations, resulting in the loss of normal immune induction (anergy).

The detailed pathogenesis of MAIDS is not completely understood and there are gaps in the sequence of events. MAIDS virus alone can produce disease even though it cannot replicate. The virus mainly infects B lymphocytes and expresses a single protein, a variant form of $Pr60^{gag}$ (a 60 kD protein encoded by the *gag* gene that produces the internal structural proteins of the virus particle), which is both necessary and sufficient for the production of MAIDS. $Pr60^{gag}$ acts by binding to the plasma membrane, but it is not clear how binding leads to the hyperproliferation of B lymphocytes. B lymphocyte proliferation initiates immune activation, including proliferation of CD4+ T lymphocytes, but the cytokine pathways responsible for activation are not thoroughly delineated. Finally, the mechanisms by which immune activation interferes with normal immune induction – producing functional immune suppression – remain to be dissected.

VIRAL INFECTIONS OF HUMANS ASSOCIATED WITH IMMUNOSUPPRESSION

There are a number of human viruses that cause immunosuppression of some degree (see Table 8.5). Several of the most prominent examples are discussed below.

Measles

Measles is the prototype of an acute virus infection that produces global immunosuppression. Figure 8.1 shows the tuberculin skin test response before, during and after measles virus (MV) infection. Suppression develops during the 10-day incubation period and is most pronounced during the clinical phase of disease that lasts about one week. Recovery occurs within 3–4 weeks after the rash and the tuberculin response is essentially normal by one month after the acute illness. During the period of measles-induced immune suppression there is an increased susceptibility to other infections, a transient potential for exacerbation of chronic infections such as tuberculosis and remission of autoimmune diseases such as juvenile rheumatoid arthritis and the nephrotic syndrome.

The mechanisms of measles-induced suppression are complex and probably involve at least three different cell types and pathways:

1. Dendritic cells and monocytes are infected and their antigen-presenting activity is severely compromised, with reduced development, proliferation and ability to traffic. As mentioned above, this is associated with an aberrant response to type 1 IFN leading to inhibition via a STAT2-dependent signaling pathway.
2. Circulating T lymphocytes (CD4+ and CD8+) are decreased by about 50%, due to both apoptosis and failure to proliferate. Relatively few monocytes and lymphocytes are infected, which suggests that indirect mechanisms play a role in lymphopenia. When examined ex vivo, T lymphocytes from acutely infected patients show a decreased proliferative response to mitogens, such as phytohemagglutinin that cause most T cells to divide (Figure 8.8).
3. There are aberrant cytokine responses; Il-4 and Il-10 production is increased (skewing the immune response from T_H1 to T_H2 and reducing macrophage activation) and Il-12 and TNFα production is decreased.

Cell cultures of monocytes and lymphocytes can be infected with MV and the infection alters their physiological responses, although it does not kill the cells. When CD4+ T lymphocytes are exposed to cells (or MV) expressing the F (fusion) and H (hemagglutinin) proteins, the T cells lose their ability to respond to mitogens

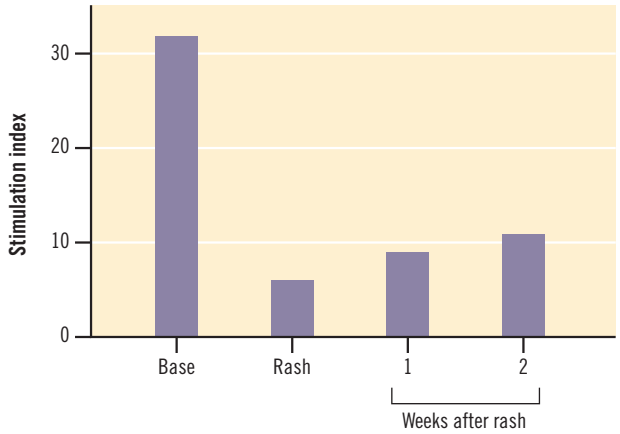

FIGURE 8.8 Measles virus infection abrogates the proliferative response of T lymphocytes to mitogens. Peripheral blood mononuclear cells were cultured with mitogen (phytohemagglutinin, a lectin that stimulates most T lymphocytes to divide) and their incorporation of ^{3}H thymidine determined in comparison with replicate cultures absent mitogen. After Hirsch RL, Griffin DE, Johnson RT *et al.* Cellular immune responses during complicated and uncomplicated measles virus infections of man. *Clinical Immunology and Immunopathology* 1984, 31: 1–12, with permission.

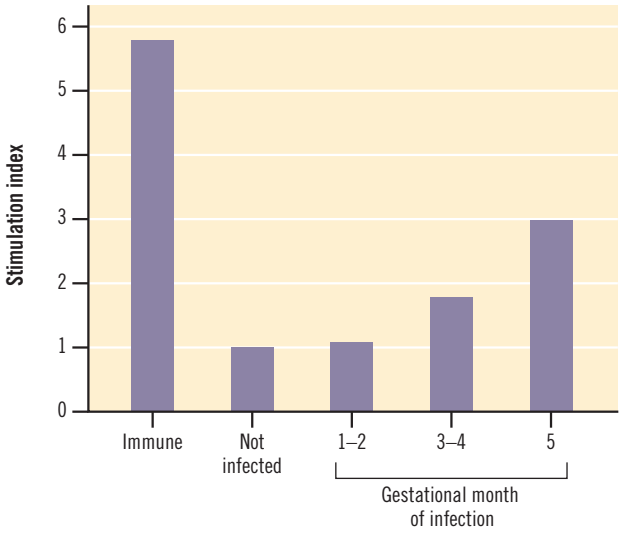

FIGURE 8.9 Defective cell-mediated immune responses associated with fetal infection. Children with the congenital rubella syndrome who had been infected at different gestational ages are compared with children infected postnatally (immune column) and with uninfected children in a lymphocyte proliferation assay using rubella virus as antigen. The children with congenital rubella syndrome exhibit reduced proliferation responses even though they were tested up to 10 years of age. After Buimovici-Klein E, Lang PB, Ziring PR, Cooper LZ. Impaired cell-mediated immune response in patients with congenital rubella: correlation with gestational age at time of infection. *Pediatrics* 1979, 64: 620–626.

even at a ratio of infected to uninfected cells of 1:100. Inhibition is not mediated by soluble factors and requires contact between infected and uninfected cells. The effect entails both F and H proteins and cleavage of the F_0 to F_1 and F_2 peptides. Binding of the F/H complex to a MV receptor on the lymphocyte has been reported to reduce the ability of T lymphocytes to respond to Il-2, associated with inhibition of the Akt kinase signaling pathway.

In summary, it appears that the function of antigen-presenting cells (dendritic cells and macrophages), helper cells (CD4+ T lymphocytes) and effector cells (CD8 T lymphocytes) are all compromised during measles.

Rubella

Rubella (German measles) is an example of a virus that only causes immunosuppression in utero or infancy. In children or adults, rubella is an acute infection, which produces a brisk immune response that clears the virus within a few weeks. If primary infection occurs during pregnancy, the virus can cross the placenta and infect the fetus. Fetal infections – which often produce the congenital rubella syndrome, i.e. developmental malformations – usually persist throughout pregnancy and are not cleared until about 6–12 months of age. Infants or children with the congenital rubella syndrome exhibit markedly diminished cellular immune responses to rubella virus antigens even when tested many years after in utero infection (Figure 8.9). However, such infants do raise brisk antibody responses to rubella. IgM anti-rubella antibodies are present at birth and wane from 6 to 12 months, concomitant with the clearing of the virus, while maternal IgG antibodies are predominant from birth to 6 months and are then replaced by the infant's anti-rubella IgG that is long lasting. It appears that gestational infection with rubella virus leads to a suppression of the cellular but not the humoral immune response and that the failure to clear virus is associated with suppression of cellular immunity.

Human immunodeficiency virus (HIV) and AIDS

HIV is the best studied example of a human virus infection associated with immunosuppression. The pathogenesis of AIDS is the subject of Chapter 14 and the following sketch focuses on the mechanisms of immunosuppression. The clinical features of AIDS (acquired immunodeficiency syndrome) are associated with the loss of host immune defenses and the occurrence of opportunistic infections (OIs) or neoplasms. Most OIs, such as *Pneumocystis carinii* (a fungal lung infection), cytomegalovirus retinitis, or tuberculosis, pre-exist AIDS and represent activation of persistent infections that are latent or contained. Some AIDS-associated neoplasms, such as lymphoma, are seen in immunologically intact individuals but at a higher incidence in AIDS, while others, such as Kaposi's sarcoma, are rare except in patients with AIDS. Cellular immune responses to opportunistic infectious agents are reduced in patients with AIDS compared to normal subjects while antibody titers are generally maintained until late in the illness. When patients with OIs receive highly active antiretroviral treatment (HAART), a remarkably rapid clearing of the OIs is often seen, implying a return of the effector component of cellular immunity.

The CD4 molecule is the primary receptor for HIV and the virus mainly infects T lymphocytes and monocytes (and macrophages), the two main cell types that express CD4. HIV is cytolytic for T lymphocytes, but not for macrophages, and immunodepression in AIDS is due

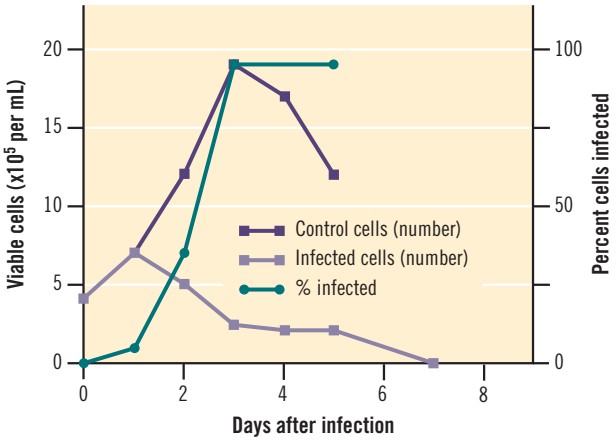

FIGURE 8.10 Some viruses exert their immunosuppressive effect by destroying specific cells of the immune system. In this example, HIV destroys CD4+ T lymphocytes in cell culture. A T cell line (MT-4) derived from cord blood was infected with a high multiplicity of HIV and the culture followed to determine the number of viable cells and the proportion infected. The infection spread to 100% of cells in about 3 days, with rapid destruction of the culture compared to an uninfected sister culture. After Harada S, Koyanagi Y, Yamamoto N. Infection of HTLV-III/LAV in HTLV-I-carrying cells M-2 and MT-4 and application in a plaque assay. *Science* 1985, 229: 563–566, with permission.

in part to the loss of CD4+ T lymphocytes (Figure 8.10), while monocyte function is not severely compromised. In HIV-infected persons, the reduction of the CD4+ lymphocyte population is a complex process that usually occurs gradually over many years. At any time, only a small proportion of CD4+ cells are infected and cytolysis is limited to those infected T cells that are actively proliferating, while the virus is relatively latent in quiescent infected T cells. In the first months or years after infection, the destruction of CD4+ lymphocytes increases, but there is a compensatory increase in the production of new T cells from the bone marrow, so that total CD4+ lymphocyte concentration in peripheral blood does not decrease. Eventually, destruction exceeds production and the concentration of circulating CD4+ cells decreases. When CD4+ cell counts drop below a critical threshold, patients begin to develop opportunistic infections. In uninfected normal humans, the concentration of CD4+ lymphocytes in peripheral blood is in the range 1000–1500 per μl and opportunistic infections are associated with CD4+ cell levels less than 100–400 per μl.

CD4+ T lymphocytes act as 'helper' cells for the induction of both cellular (CD8+ cytolytic T cells) and humoral (antibody) immune responses (see Chapter 6). Thus, HIV-infected patients are impaired in their ability to develop de novo immune responses and are particularly susceptible to new infections against which they have no prior immunity. In addition, persistent latent infections, such as tuberculosis, that are contained by cellular immunity, may reactivate. Mature humoral responses that pre-date the onset of AIDS tend to remain relatively intact because long-lived plasma cells continue to produce antibody without the need for helper T cells.

These observations appear to represent a paradox. Clinically, AIDS usually presents as the activation of pre-existing infections, representing a failure of the effector limb of the cellular immune response – mediated by CD8 cells – to contain long-standing latent OIs. But CD4+ cells – not CD8+ cells – are the direct target of the virus. Likely, the failure of the effector limb of the immune response is partly due to the loss of the helper functions provided by CD4+ cells and partly to dysregulation of CD8 cells, a matter that is discussed in more detail in Chapter 14.

DETERMINANTS OF IMMUNOSUPPRESSION

The ability of a virus to initiate immunosuppression depends upon some rather subtle interactions with the lymphoreticular system. Therefore, it is not surprising that a number of variables such as virus strain, virus dose, route of injection, age of the host and extraneous immunosuppression will determine whether a virus induces an immunogenic or an immunosuppressive response.

Virus strain: lymphocytic choriomeningitis virus (LCMV)

The difference between strains of LCMV has already been discussed. In adult mice, the Armstrong strain of LCMV causes an acute short-term rapidly cleared infection and is not immunosuppressive, while the clone 13 variant causes a persistent infection associated with immunosuppression and these differences are associated with the ability of clone 13 to replicate in and destroy macrophages and dendritic cells, which act as 'professional' antigen-presenting cells. The immunosuppressive property of clone 13 has been mapped to two amino acids, one each in the viral envelope protein and the viral polymerase.

Virus strain: simian immunodeficiency virus (SIV)

SIV, like HIV, produces immunodepression by depleting CD4+ T lymphocytes. As described above, there is an unstable equilibrium between destruction and replacement of CD4+ lymphocytes. SIV strains that are engineered to delete their *nef* gene, a regulatory protein, maintain their replicative capacity in cultured CD4+ lymphocytes, but are much less immunosuppressive in rhesus macaques than wildtype SIV. The differences are due to a function of the nef protein that down modulates the expression of MHC class I molecules on the surface of infected lymphocytes (see Chapter 4), so that infected cells are less susceptible targets for CTL attack. Although this has no effect in cell culture, it does have a dramatic effect on virus titers in vivo that, in turn, influence pathogenicity (Figure 8.11).

Age and immunosuppression

The LCMV model also illustrates that the mechanism of immunosuppression is different in newborn and adult mice. LCMV infection of adult mice, described above,

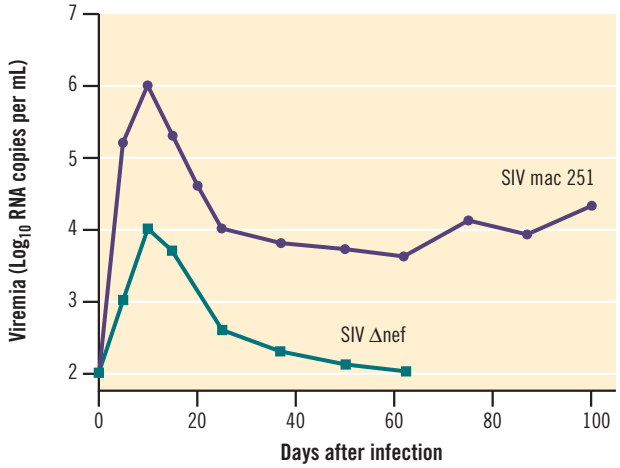

FIGURE 8.11 Viral 'accessory' genes can enhance infection by suppressing the antiviral immune response. SIV mac 251 wildtype is compared with a mutant engineered to delete the *nef* gene, a regulatory gene that reduces expression of MHC class I molecules on the plasma membrane, permitting escape of virus-infected cells from immune attack. The nef-deleted virus is attenuated and does not replicate as effectively as wildtype SIV. After Connor RI, Montefiori DC, Binley JM *et al.* Temporal analyses of virus replication, immune responses, and efficacy in rhesus macaques immunized with a live attenuated simian immunodeficiency virus vaccine. *Journal of Virology* 1998, 72: 7501–7509, with permission.

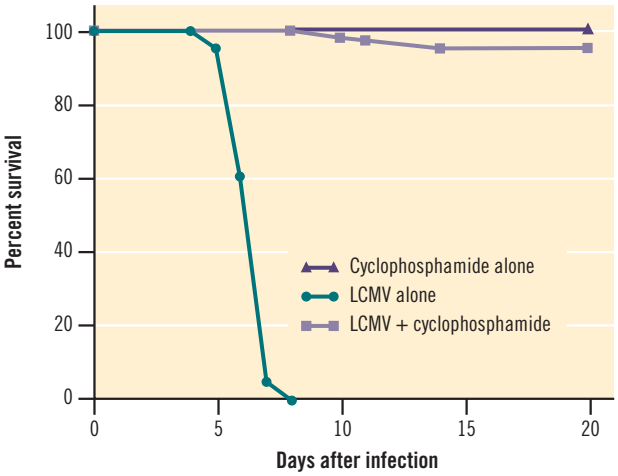

FIGURE 8.12 Transient chemical or physical immunosuppressive treatment can initiate prolonged immunosuppression with certain viruses. Mice injected intracerebrally with LCMV (Armstrong strain) raise a brisk CTL response that causes a lethal choriomeningitis. If the animals are treated with a single dose of cyclophosphamide (150 mg per kg) on day 3 of infection, most of them survive. In the survivors, the antiviral CTL responses are minimal and the animals develop persistent infections. After Gilden DH, Cole GA, Nathanson N. Immunopathogenesis of acute central nervous system disease produced by lymphocytic choriomeningitis virus. I. Cyclophosphamide-mediated induction of the virus-carrier state in adult mice. *Journal of Experimental Medicine* 1972, 135: 860–872, with permission.

can result in a 'global' immunodepression that affects responses both to LCMV and to other viruses. On the other hand, LCMV infection of newborn mice results in suppression of responses to LCMV but not to other viruses. This striking difference is illustrated in Table 8.3 and probably reflects different mechanisms of suppression. It has already been suggested that, in adult mice, clone 13 infects and destroys macrophages (and dendritic cells), thereby interfering with processing and presentation of many antigens. In newborn mice, clone 13 produces a very widespread infection of many organs and tissues. The virus is treated as a 'self' antigen with the induction of 'tolerance' by clonal deletion of LCMV-specific T lymphocytes, by the same mechanism that produces tolerance to self antigens.

Other determinants of immunosuppression

A number of other variables such as the dose of virus, the route of injection and chemical or physical immunosuppressive treatments, can influence whether a virus causes immunosuppression. In some circumstances, a large virus dose will produce 'high dose tolerance' where a small inoculum will initiate an immunogenic response (see Table 8.4). Intravenous virus injection may initiate immunosuppression while intracerebral infection is immunogenic. Transient treatment with X-irradiation or the immunosuppressive drug, cyclophosphamide, can convert an immunogenic infection into an immunosuppressive one (Figure 8.12).

REPRISE

Most viral infections induce immune responses to the viral antigens but, under some circumstances, a virus can be immunosuppressive. Virus-induced immunosuppression is often 'global', affecting the response to many antigens but, in some instances, suppression is limited to the infecting agent. Immunosuppression can be induced through several mechanisms, including:

1. destruction of dendritic cells (or other antigen presenting cells) or subsets of T lymphocytes
2. infection of fetal or newborn animals leading to tolerance
3. perturbation of cytokine homoeostasis
4. viral proteins that may act as virokines (simulating cytokines) or viroceptors (simulating cellular receptors).

Virus-induced immunosuppression reflects the delicate balance between host and parasite and is influenced by a number of variables such as strain of virus, dose and route of infection and immunocompetent status of the host at the time of infection.

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PART III

Virus–Host Interactions

Chapter 9

CHAPTER CONTENTS

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REPRISE

FURTHER READING

Viral Virulence

Neal Nathanson and Francisco Gonzalez-Scarano

Virulence (or pathogenicity) refers to the ability of a virus to cause disease in an infected host. Virulence is a variable biological property of the virus and clones or strains of a single virus can differ widely in their pathogenicity. Virulence is central to the study of pathogenesis, since variants of a single virus will exhibit different patterns of pathogenesis, a consideration that, if overlooked, can confound studies of pathogenesis. Furthermore, virulence has important practical implications, since avirulent (also called attenuated) variants of a virus are often used as live virus vaccines. Examples of the latter are vaccines for smallpox, poliomyelitis, measles and yellow fever.

There are two distinct yet complementary approaches to the study of virulence. Virulence can be considered a property of the virus, in which case it is possible to utilize genetic methods to define the role of specific viral genes and proteins in determining pathogenic phenotypes. This approach can be extended to the molecular and structural level since, in some instances, single amino acids can exert a

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marked influence on pathogenicity. For instance, variation in a single amino acid in the envelope of a viral protein can determine how readily it is cleaved by a cellular protease, and this property in turn influences the range of tissues that can be infected and the corresponding disease pattern.

The other approach is the comparative pathogenesis of virulent and avirulent virus strains, to elucidate the biological mechanisms underlying their phenotypes. Variation in pathogenicity must be regarded as a multidimensional phenomenon that is both quantitative and qualitative. Qualitative variation may be manifested in various ways. For instance, viral clones may exhibit differences in their tropism, so that one clone replicates well in the brain while another clone replicates well in the liver or intestinal tract. Alternatively, different clones may spread by different routes, one clone producing viremia while another clone spreads by the neural route. In some instances, viral clones will vary in the immune responses that they induce, or in their susceptibility to antibody or to cellular immune defenses. For any given expression of pathogenicity, it is possible to quantify virulence by comparing, for different viral strains, the number of infectious units required to produce a specified outcome such as mortality or other diseases parameter.

In summary, virulence is intimately intertwined with pathogenesis. On the one hand, it is important to understand the characteristics of the viral strain that will be used to characterize the pathogenesis of a given virus and to recognize that the pathogenesis might vary for another strain of the same virus. On the other hand, it is critical to specify which specific phenotype will be evaluated in order to compare the virulence of different viral strains, since relative pathogenicity depends upon the parameter that is assessed. These points are illustrated below.

MEASUREMENT OF VIRULENCE

Relative nature of virulence

Virulence is not an absolute property of a virus, but depends upon many variables, such as the viral strain, the route of infection and dose of virus and the species, age and genetic susceptibility of the host. The relative nature of virulence is illustrated in Table 9.1; it compares a virulent wildtype strain of La Crosse virus with an attenuated mutant clone (B.5) derived from the wildtype strain. Suckling mice are highly susceptible to intracerebral injection with either virus (1 plaque forming unit (PFU) will cause a fatal infection) and there is no difference between the two viruses. However, after subcutaneous injection of suckling mice, the wildtype virus retains its pathogenicity, while clone B.5 is much less virulent, because of its minimal ability to produce a viremia and reach the brain. La Crosse virus is virulent for adult mice after intracerebral injection, but clone B.5 is attenuated because of its reduced ability to replicate in neurons. After subcutaneous injection of adult mice, neither virus will cause illness at any dose, so the two viruses appear equally innocuous. To profile the differences between the virulent and attenuated viruses, it is necessary to choose a combination of host and route that is between the most susceptible end of the scale (intracerebral injection of suckling mice) and the most resistant end of the scale (subcutaneous injection of adult mice).

La Crosse virus strain	Intracerebral infection PFU per LD50		Subcutaneous infection PFU per LD50	
	Suckling mice	Adult mice	Suckling mice	Adult mice
Virulent wildtype	~1	~1	~1	$>10^7$
Attenuated clone B.5	~1	$>10^6$	$>10^5$	$>10^7$

TABLE 9.1 Virulence can be expressed as the number of infectious units (PFU, plaque forming units) required to kill 50% of the animals (ratio $\log_{10}$ PFU per LD50). The table shows a comparison of two variants of La Crosse virus, wildtype and attenuated clone B.5. This table also illustrates that virulence is a relative phenomenon and depends upon the method of assessment
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Measures of virulence

There are many measures that can be used to quantify virulence or attenuation. Most commonly, death or a constellation of symptoms and signs is used, such as paralysis (poliovirus), jaundice (hepatitis viruses), rash (measles), change in blood chemistry (hepatitis) and the like. Many viruses cause a large number of inapparent (asymptomatic) infections as well a few cases of overt illness. For such viruses, the case:infection ratio can be used as a measure of virulence. Table 9.2 compares the ratio for wildtype poliovirus to the ratio for attenuated poliovirus vaccine (OPV, oral poliovirus vaccine), showing that the vaccine virus is about 10 000-fold more attenuated than wildtype virus.

The severity of illness or the incubation period can also be used as a measure of virulence. Domestic rabbits were introduced into Australia in the early 20th century and escaped captivity to become feral. Due to a lack of natural predators, the rabbit population expanded astronomically and threatened farming in some regions of the country. To control these pests, myxoma virus, a naturally occurring poxvirus of wild rabbits (but not of domestic rabbits) was introduced and caused a rapid die off of the population. Over the following years, the virus underwent natural attenuation and a large group of isolates were compared for their degree of virulence, based on case:fatality rate and incubation period as shown in Table 9.3.

Virulence can also be assessed by the quantitation of pathological lesions in an organ that is attacked by the virus under study. Figure 9.1 shows a comparison of the central nervous system lesions caused by five neurotropic flaviviruses. Some viruses produced most severe lesions in the brain, others targeted the spinal cord and some produced similar lesions throughout the neuraxis, reflecting the multidimensional nature of virulence. Laboratory tests can be used as indirect indicators of the disease severity in a particular organ. Thus, the severity of hepatitis can be measured by the serum titer of alanine transaminase which reflects the release of intracellular proteins from dying hepatocytes and the severity of AIDS can be assessed by the blood concentration of CD4 lymphocytes.

Virus	Study period	Paralytic rate per 100 primary infections	Relative rates
Wildtype	1931–1954	0.7	~10 000
OPV	1961–1978	0.000062	1

TABLE 9.2 For viruses that have a low case:infection ratio, virulence can be expressed as the ratio of cases of disease to total infections. Poliovirus is an example since most human infections are inapparent, with <1 paralytic case per 100 infections. In this example, two strains of poliovirus are compared, wildtype and attenuated vaccine virus (OPV, oral poliovirus vaccine) which is highly attenuated but still causes a small number of cases of paralysis
After Nathanson N, McFadden G. Viral virulence, in Nathanson N (ed.), *Viral pathogenesis*, Lippincott Raven Publishers, Philadelphia, 1997.

Virulence grade	Case fatality rate (%)	Survival time (days)	Percent of isolates
I	>99	<13	4
II	95–99	14–16	18
IIIA	90–95	17–22	39
IIIB	70–90	23–28	25
IV	50–70	29–50	14
V	<50	NC	1

TABLE 9.3 Virulence can be measured as the proportion of infections that are fatal (case:fatality rate) or by the survival time until death. In this example, various strains of myxoma virus, a poxvirus of rabbits, were compared after subcutaneous inoculation
NC: not calculable. After Marshall ID, Fenner F. Studies in the epidemiology of infectious myxomatosis of rabbits. VII. The virulence of strains of myxoma virus recovered from Australian wild rabbits between 1951 and 1959. *Journal of Hygiene* 1960, 58: 485–488.

Quantitation of virulence

Virulence can be quantitated in experimental models by determining the number of infectious units required to produce a specific endpoint. For instance, calculation of the PFU per 50% lethal dose (LD50) makes it possible to distinguish different degrees of virulence as shown in Figure 9.2, which separates viruses of high and low virulence. Other semi-quantitative methods are based on the

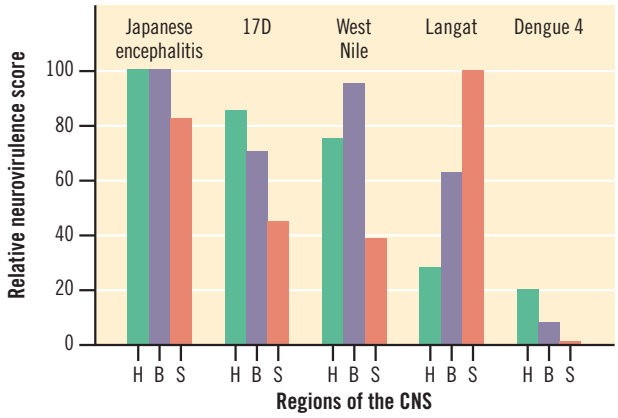

FIGURE 9.1 Pathological lesions can be used to measure virulence. In this example, monkeys were injected intracerebrally with five different neurotropic viruses and the severity of lesions in different central nervous system regions were graded using a standard scale (H: hemispheres; B: brainstem; S: spinal cord). This figure also shows that different viruses cause varying lesion profiles, so that lesion severity is a qualitative as well as a quantitative phenomenon. After Nathanson N, Gittelsohn AM, Thind IS, Price WH. Histological studies of the monkey neurovirulence of group B arboviruses. III. Relative virulence of selected viruses. *American Journal of Epidemiology* 1967, 85: 503–517.

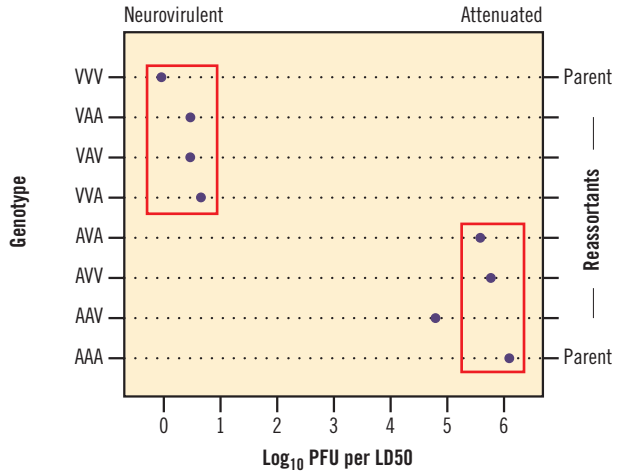

FIGURE 9.2 Virulence can be expressed in a quantitative manner by determining the number of infectious units to produce a given disease phenotype. In this example, different virus clones were titrated for infectivity in tissue culture and for neurovirulence by intracerebral injection of adult mice and the ratio PFU per LD50 was calculated. Bunyaviruses are tri-segmented and the three letters refer to the large, middle and small gene segments: A, attenuated; V, virulent. An attenuated clone was crossed with a neurovirulent clone and viruses of all eight possible genotypes were tested in a quantitative assay by intracerebral titration in adult mice. The neurovirulent phenotype segregated with the L RNA segment that encodes the viral polymerase. After Endres MJ, Griot C, Gonzalez-Scarano F, Nathanson N. Neuroattenuation of an avirulent bunyavirus variant maps to the L RNA segment. *Journal of Virology* 1991, 65: 5465–5470.

severity of pathological lesions (Figure 9.1), the case:infection ratio (Table 9.2) and the case:fatality rate (Table 9.3). Various empirical systems have been developed to compare the virulence of strains of a specific virus, based on the pathogenesis of individual virus diseases. For instance, strains of Marek's disease virus, an oncogenic α-herpesvirus of chickens, are tested for their ability to cause lymphoproliferative lesions in a specific inbred strain of chickens that has been vaccinated against MDV.

EXPERIMENTAL MANIPULATION OF VIRULENCE

To study virulence, it is necessary to possess viral variants that differ in their pathogenicity. For some viruses, naturally occurring isolates exhibit marked differences in virulence. For instance, type 1 and type 3 reoviruses differ sharply in tropism and yet are genetically so similar that reassortants (containing a mix of gene segments from types 1 and 3 viruses) can be readily obtained. For many viruses, however, it is necessary to use laboratory manipulation to obtain virus clones that are attenuated relative to the wildtype viruses isolated from nature. A number of procedures have been utilized to obtain attenuated viral variants.

Passage in animals

In the pioneering days of virology, viruses were often maintained by serial passage in animal hosts. It was found that pathogenicity would change during the course of passage and this adventitious finding was exploited to obtain viruses of different pathogenicity. In general, during repeated animal-to-animal transmission a virus will adapt to replicate optimally and become virulent under the conditions of passage. Yellow fever, a flavivirus, produces fatal hepatitis in monkeys; if passaged intracerebrally in mice it will become highly neurovirulent for mice but will lose its ability to cause hepatitis in monkeys. However, in nature, where viruses have adapted to specific hosts, virulence may vary from high (rabies in raccoons or foxes, smallpox in humans) to low (Ebola virus or severe acute respiratory syndrome (SARS) coronavirus in bats).

Passage in cell culture

With the advent of cell culture, viruses were usually maintained by cell culture passage and it was soon observed that serial transmission would alter the biological phenotype, often reducing virulence for animals or humans. This observation was exploited in the deliberate search for attenuated variants that could be used as prophylactic vaccines. A typical example of the effect of passage upon virulence is shown in Table 9.4. An uncloned series of La Crosse virus strains were each passaged 25 times in BHK-21 cells and the resulting uncloned viruses were tested for neurovirulence in mice. Of the 10 passage lines, one (RFC/B) appeared to be the most attenuated and 10 clones from this virus stock were tested in mice. Of these 10 clones, one (RFC/B.5) appeared to be the most attenuated and was selected for further study. This experiment illustrates several underlying principles:

- Apparently identical passage lines can yield virus stocks differing in their virulence.
- An RNA virus stock represents a 'swarm' of virus clones of different phenotypes.

Passage line	Mortality (dead/tested)
Each virus passed 25 times, then tested in mice	
La Crosse/original, pass A	5/5
La Crosse/original, pass B	3/5
La Crosse p10, pass A	3/5
La Crosse p10, pass B	4/5
La Crosse/pp31, pass A	5/5
La Crosse/pp31, pass B	4/5
Tahyna/181–57, pass A	5/5
Tahyna/181/57, pass B	5/5
La Crosse/RFC, pass A	1/5
La Crosse/RFC, pass B	*0/5*
RFC/B clones tested in mice	
1	5/5
2	4/5
3	5/5
4	3/5
5	*0/5*
6	4/5
7	2/5
8	1/5
9	5/5
10	1/5

TABLE 9.4 The effect of passage in cell culture upon the virulence of La Crosse bunyavirus. A number of different strains of La Crosse virus were passed 25 times in BHK-21 cells and were then tested for virulence by intracerebral injection of 100 PFU into adult mice. One of these passaged lines (RFC/B) appeared relatively attenuated and 10 plaques were further tested by intracerebral injection of 10 000 in adult mice. Plaque B.5 was selected as a highly attenuated clone
After Endres MJ, Valsamakis A, Gonzalez-Scarano F, Nathanson N. Neuroattenuated bunyavirus variant: derivation, characterization, and revertant clones. *Journal of Virology* 1990, 64: 1927–1933.

- Passage probably selects for virus clones already in the population which grow preferentially and thus alter the phenotype of the virus swarm.

Passage and viral phenotype

Historically, the failure to recognize the influence of passage upon the biological phenotype has led to some important errors in virological research. Thus, intracerebral passage of poliovirus in monkeys leads to selection of variants that are highly neurotropic but have lost much of their infectivity and pathogenicity when administered by the oral (natural) route. Studies with neuroadapted poliovirus resulted in the mistaken conclusion that poliovirus was not an enterovirus but was naturally transmitted by the intranasal route and this misapprehension led to trials of nasal astringent sprays as a method to protect children against paralysis.

More recently, passage of HIV in T cell lines selected for laboratory variants that differed from wildtype virus in the exclusive use of the CXCR4 coreceptor, inability to infect macrophages and ability to plaque in MT-2 cells (a T cell line). When used for serological assays, the adapted viruses are readily neutralized by sera from patients naturally infected with HIV. These findings resulted in the mistaken conclusion that HIV could be readily neutralized and the prediction that it would be relatively easy to develop a prophylactic vaccine. Once it was recognized that viral isolates only maintained their natural phenotype if passaged in primary blood mononuclear cells (PBMC), it became clear that wildtype HIV isolates were very resistant to neutralization, presenting a daunting challenge for vaccine development.

Selection of attenuated variant viruses

There are a number of methods that have been used to enhance the selection of attenuated virus variants from an uncloned virus stock.

Temperature sensitive mutants

Wildtype viruses will replicate well at the standard temperature of 37°C and at temperatures up to 40°C (most cell cultures do not thrive above 40°C). Temperature sensitive (ts) variants, on the other hand, replicate well at 37°C but poorly if at all at 40°C. It is relatively easy to select for ts variants and they will often exhibit an attenuated phenotype when tested in animals. The attenuated phenotype is not due to the body temperature of the test animal (which is usually around 37°C, a permissive temperature for ts mutants). Instead, ts mutants usually have a restricted range in host tissues relative to wildtype virus. Figure 9.3 illustrates these points for the attenuated RBC/B.5 clone whose derivation is described in Table 9.4. Clone B.5 replicates quite well in BHK-21 cells at 37°C but hardly at all at 40°C, whereas wildtype La Crosse virus replicates equally well at both temperatures. Furthermore, clone B.5 replicates very poorly in mouse neuroblastoma cell cultures (NA cells) even at 37°C, which is further evidence of its host restriction. Finally, clone B.5 replicates very poorly after intracerebral injection by comparison with neurovirulent wildtype La Crosse virus.

Cold adapted variant viruses

Another method for the selection of attenuated mutants is passage at a low temperature, such as 25°C, about the lowest temperature at which mammalian cell cultures can be maintained. Table 9.5 shows an example of a cold adapted influenza virus, compared with its wildtype parent virus stock. The cold adapted variant is temperature sensitive and exhibits restricted pneumotropism in ferrets, an animal that develops severe pneumonia after intranasal infection with wildtype human influenza viruses.

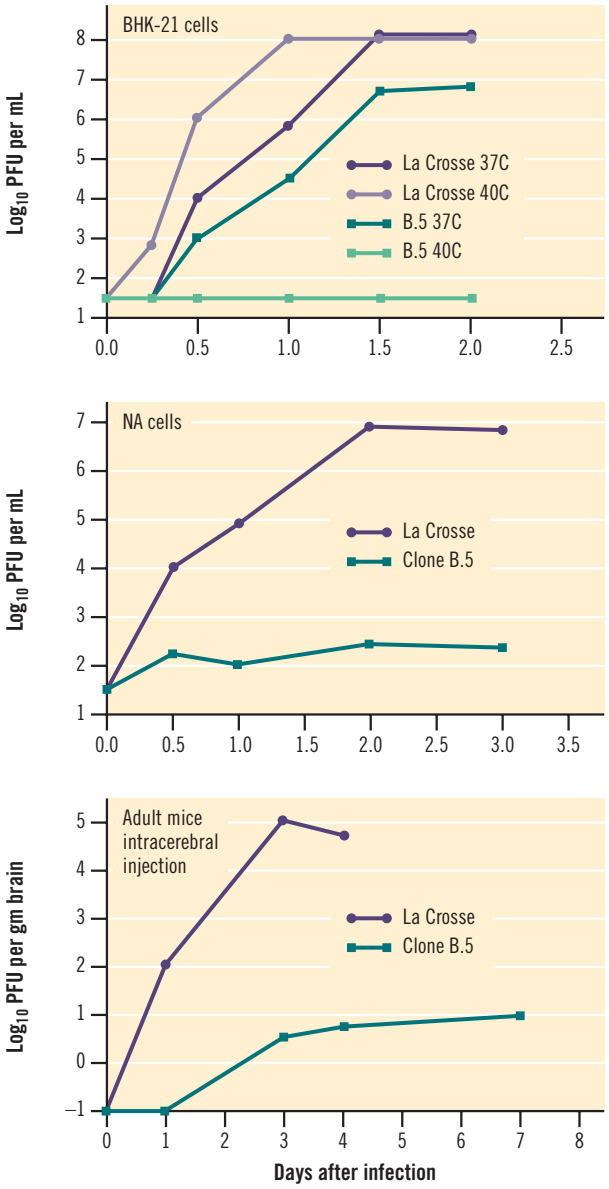

FIGURE 9.3 Phenotype of an attenuated variant virus showing its temperature sensitivity, restricted host range and reduced virulence in animals. La Crosse virus, clone RFC/B.5 is temperature sensitive (upper panel), restricted in its ability to replicate in neuroblastoma cells (middle panel) and had reduced neurovirulence after intracerebral injection of 700 PFU in adult mice (lower panel). After Endres MJ, Valsamakis A, Gonzalez-Scarano F, Nathanson N. Neuroattenuated bunyavirus variant: derivation, characterization and revertant clones. *Journal of Virology* 1990, 64: 1927–1933.

Monoclonal antibody resistant (MAR) viruses

Monoclonal antibodies with neutralizing activity can readily be obtained for most viruses. When an uncloned RNA virus stock is treated with a neutralizing monoclonal antibody, there is a residual fraction – 10^{-4} to 10^{-6} – that resists neutralization. The resistant fraction yields clones that are resistant to the selecting monoclonal antibody and probably represent naturally occurring point mutants (mutants with a single nucleotide change) which, for a single nucleotide, tend to occur at the rate of about 10^{-5} in most RNA virus populations. When tested in animals, some MAR mutants are attenuated and these have been used to study the molecular basis of attenuation since their phenotype can be mapped to a specific viral protein (the virus attachment protein) and often to a single amino acid in that protein.

Character	Not adapted	Cold adapted
Replication (PFU per ml) at		
37°C	$10^{7.3}$	$10^{7.3}$
30°C	$10^{4.6}$	$10^{6.4}$
25°C	$10^{2.0}$	$10^{6.5}$
Temperature sensitive at 40°C	No	Yes
Virulence for ferrets (intranasal infection)	Yes	No

TABLE 9.5 Selection of attenuated virus by cold adaptation. An influenza virus was passaged 6 times in cell culture at 25°C and its properties compared with the non-adapted parent virus from which it was derived
After Maassab HF, De Border DC. Development and characterization of cold-adapted viruses for use as live virus vaccines. *Vaccine* 1985, 3: 355–369.

Mutagenized viruses

To increase the likelihood of an altered phenotype, it is possible to use irradiation or chemical agents to mutagenize a virus stock, following which it can then be tested for altered phenotype. Although mutagenesis is an effective way to increase the frequency of viruses with an altered phenotype, it usually introduces multiple mutations, making it difficult to determine the viral gene or protein responsible for the altered phenotype. In contrast, clones with altered phenotypes obtained from non-mutagenized viral stocks are more likely to possess a limited number of genetic changes.

With the introduction of modern molecular genetics into virology, for many virus families it has become relatively easy to alter the viral genome in a controlled manner. Genetic changes that can be deliberately introduced include point mutations that alter function of individual proteins, or inactivation of non-essential viral genes by introduction of stop codons or deletions of substantial gene segments. Viruses with 'designer' mutations can then be compared with the parent clones from which they were derived.

Choice of attenuated viruses for study

Before investing effort in characterization of attenuated mutants, it is worthwhile considering several points:

- First, it is important to select viruses that are as genetically as pure as possible. This is usually accomplished by repeated plaque purification of a viral clone prior to study.
- In addition to biological cloning, genetic cloning is now available for many viruses and provides an enhanced level of consistency. Genetic clones are particularly important if the virus is a candidate for use as an attenuated vaccine where maximal genetic stability is critical.

- Second, if a clone is to be used for determination of the genetic basis of viral phenotypes, it is preferable to use variant viruses that are likely to have a minimal number of mutations relative to the parent virus that will be used for comparison.
- Finally, the most informative variants are host range mutants, i.e. viruses that can replicate well in reference permissive cell cultures but are restricted in animals or in specialized cell cultures (see Figure 9.3). Attenuated viruses that are 'wimpy', i.e. are unable to replicate well in any cellular substrate do not provide much information about the properties of the virus that determine its in vivo virulence or attenuation.

COMPARATIVE PATHOGENESIS OF VIRULENT AND ATTENUATED VIRUSES

The sequential steps in viral pathogenesis are described in Chapter 2. In theory, two virus clones could differ at any of these steps, thereby affecting virulence or attenuation. There are examples of viruses that differ in their infectivity at the portal of entry, in their ability to disseminate, or in their replicative capacity in target organs or tissues. Also, pathogenicity may be qualitative rather than quantitative since viral clones may differ in their relative tropism for different tissues or organs. Finally, in some instances, pathogenicity is determined by the ability to induce or evade host defenses such as the immune response.

Portal of entry

Many respiratory viruses are naturally somewhat 'cold adapted' since they can replicate in the nasal mucosa and upper respiratory tract where the temperature is a few degrees lower than core body temperature. Those respiratory viruses, like influenza, that cause severe disease usually can also replicate well in the lower respiratory tract where the temperature is closer to 37°C. When influenza virus is cold adapted (see Table 9.5), it can no longer replicate well in the lower respiratory tract; such attenuated variants are being used as vaccines.

Viremia

As described in Chapter 2, most systemic viruses spread via the blood. If viral strains differ in their viremogenicity (the duration and titer of viremia), this may alter their ability to reach critical target organs and thereby influence virulence. Poliovirus is an enterovirus that is basically innocuous unless it invades the central nervous system where it destroys anterior horn cells (neurons in the spinal cord that innervate striated muscle) thereby producing a lower motor neuron flaccid paralysis. Poliovirus strains vary in the degree of viremia that they produce and this correlates with their paralytogenicity after extraneural infection (Figure 9.4).

The extent of viremia is usually an indication of the ability of the virus to replicate in cells that shed virus into the circulation. In those instances where the cellular source of viremia is known, it may be possible to correlate viremogenicity with replication in a specific organ or tissue. An example is shown in Figure 9.5 that compares two bunyaviruses. After subcutaneous injection in suckling mice, La Crosse virus can replicate well in striated muscle, produces a considerable viremia, invades the brain and is lethal. Tahyna virus strain 181/57 replicates poorly in striated muscle, fails to produce viremia and does not kill suckling mice after subcutaneous injection.

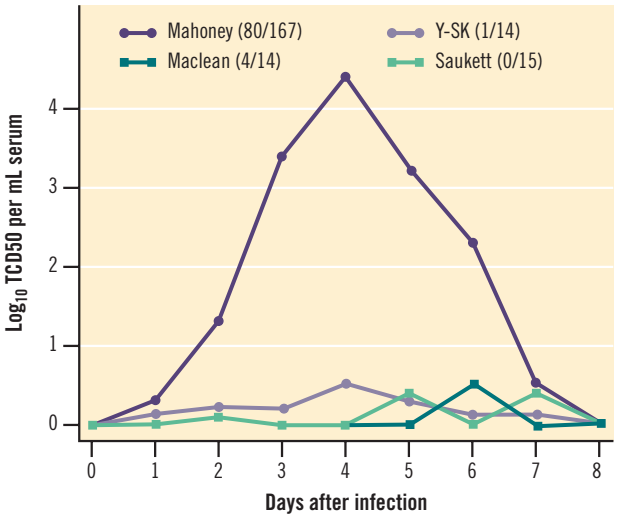

FIGURE 9.4 Viremia potential can be a determinant of pathogenicity. In this example, different wildtype strains of human poliovirus were injected intravenously in cynomolgus monkeys. The different isolates varied in the level of viremia produced and the Mahoney strain, the most viremogenic, also caused the greatest frequency of paralysis. After Bodian D. Viremia in experimental poliomyelitis. I. General aspects of infection after intravascular inoculation with strains of high and low invasiveness. *American Journal of Hygiene* 1954, 60: 339–357.

Neural spread

Some viruses spread along neural pathways rather than by viremia (see Chapter 2) and neurally spreading viruses can also be attenuated. Rabies virus is a good example of an 'obligatory' neurotrope for which attenuated strains have been derived for use as live vaccines. Vaccine strains of rabies virus, obtained by passage of wildtype virus in non-neural cultured cells, show a marked reduction in their virulence when tested by intracerebral injection in mice. The attenuated phenotype is maintained on passage in non-neural BHK-21 cells but reverts to greater virulence on passage in cultured neural cells or in the brains of suckling mice (Table 9.6). This suggests that attenuated strains probably include some virulent virus clones that are rapidly expanded in cultures where they have a growth advantage.

The basis for the attenuation of rabiesviruses has been investigated using MAR (monoclonal antibody resistant) variants selected by growing wildtype virus in the presence of monoclonal antibodies. Some of the MAR variants have markedly reduced virulence in mice and these have been studied to elucidate the mechanisms of attenuation. In one set of investigations, MAR variants with a mutation at amino acid 333 of the envelope glycoprotein were found to be attenuated; the virulent phenotype was associated with arginine or lysine (both

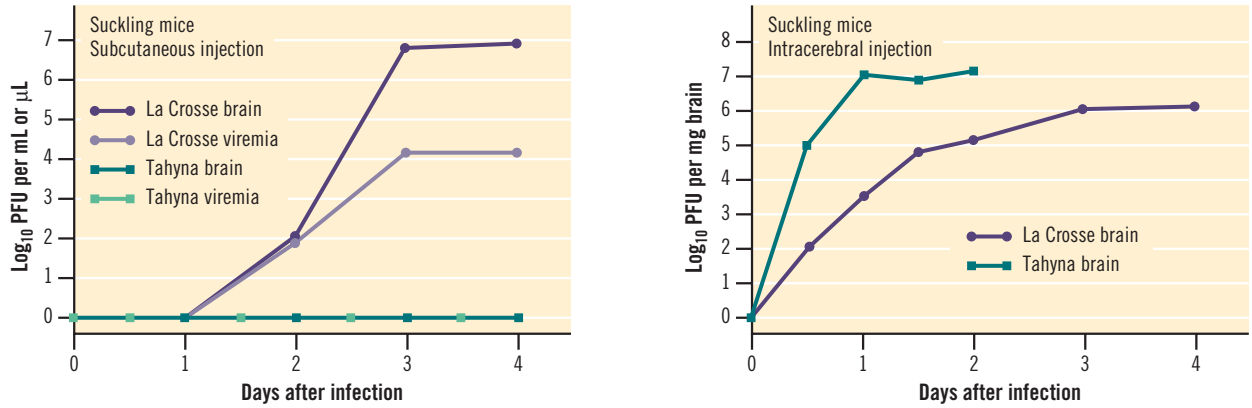

FIGURE 9.5 Virulence after parenteral virus infection can be determined by the ability of a virus to replicate in a peripheral tissue that serves a source for viremia. This example compares two bunyaviruses, La Crosse virus, which is fatal for suckling mice after subcutaneous infection and Tahyna virus strain 181/57, which produces an inapparent infection after subcutaneous infection. Left panel: La Crosse virus produces a viremia, reaches the target organ (central nervous system) and replicates to high titer in the brain. Tahyna virus fails to produce viremia and does not invade the brain. Right panel: on intracerebral injection of 700 PFU, Tahyna virus replicates to a higher titer than La Crosse virus, indicating that its peripheral attenuation was not due to a reduction in its ability to replicate in the target organ but rather to its inability to produce a viremia after subcutaneous injection. After Janssen R, Gonzalez-Scarano F, Nathanson N. Mechanisms of bunyavirus virulence: comparative pathogenesis of a virulent strain of La Crosse and an attenuated strain of Tahyna virus. *Laboratory Investigation* 1984, 50: 447–455.

Passage history HEP Flury stock	PFU per LD50	Neurovirulence (relative to HEP Flury virus)
None	>500 000	1
BHK-21 cells non-neural (5 passages)	>3 000 000	~6
NA cells neural (3 passages)	500	>1000
Suckling mouse intracerebral (4 passages)	2000	>250

TABLE 9.6 The virulence phenotype of viruses that spread by the neural route can be altered by conditions of passage. In this study, the HEP (high egg passage) Flury strain, a neuroattenuated strain of rabies virus used as a vaccine, was passed in neural cells (NA cells, a line derived from a neuroblastoma), non-neural cells (BHK, baby hamster kidney cell line) or in suckling mouse brain. The resulting stocks were titrated intracerebrally in 4-week-old mice and in BHK cells to determine PFU per LD50 (50% lethal dose)
After Clark HF. Rabies viruses increase in virulence when propagated in neuroblastoma cell culture. *Science* 1978, 199: 1072–1075.

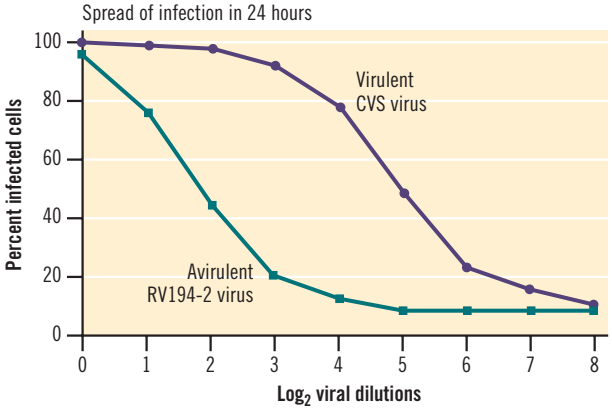

FIGURE 9.6 Attenuation of virus that spreads by the neural route correlates with the spread in cultured NA (neuroblastoma) cells. This graph compares virulent rabies virus strain CVS (challenge virus standard) with the attenuated MAR mutant RV194-2. The attenuated virus shows marked reduction in its ability to spread in neuroblastoma cells relative to the virulent virus, but both viruses spread equally fast in BHK-21 cells (data not shown). After Dietzschold B, Wiktor TJ, Trojanowski JQ *et al*. Differences in cell-to-cell spread of pathogenic and apathogenic rabies virus in vivo and in vitro. *Journal of Virology* 1985, 56: 12–18.

positively charged amino acids), while the attenuated phenotype was associated with glycine, glutamine or methionine (uncharged).

Comparative pathogenesis studies showed that after intramuscular injection, two of these attenuated variants, RV194-2 and Av01, spread into the central nervous system at about the same rate as the virulent parent virus, but once disseminated did not spread to contiguous neurons as rapidly as the virulent virus. Primary dissemination involved trans-synaptic transmission to only two or three sequential neurons, following which the attenuated viruses failed to spread further. Furthermore, when the same viruses were compared after intraocular injection, the virulent virus utilized several different neural pathways (sensory and motor) while the avirulent virus was restricted to a single pathway, indicating that attenuation involved qualitative as well as quantitative differences. Tissue culture studies showed that both virulent rabiesvirus and attenuated RV194-2 spread with equal speed through non-neural BHK-21 cells, but that they differed when used to infect NA neuroblastoma cells. The avirulent virus spread more slowly than the virulent rabiesvirus, possibly due to quantitative differences in entry into neurons (Figure 9.6).

Target organ

Variants of a virus may differ in their virulence for a target organ, such as the lung, liver or central nervous

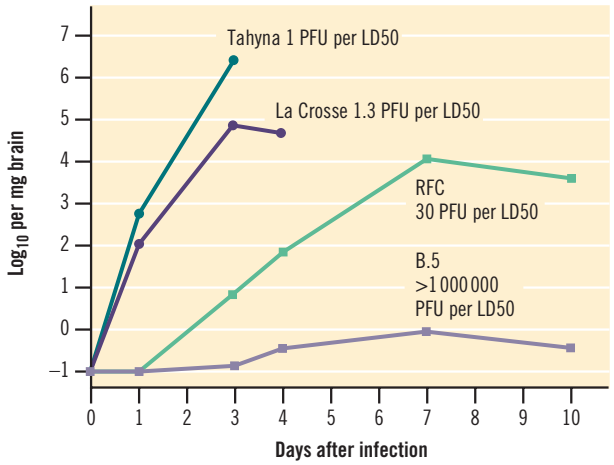

FIGURE 9.7 Relative pathogenicity for the central nervous system of four California serogroup bunyaviruses. Adult mice were injected intracerebrally with 700 PFU of different viruses each of which replicated at a different rate, consistent with the quantitative differences in their virulence expressed as PFU per LD50. Neurovirulence is distinct from neuroinvasiveness; for instance, Tahyna virus, the most neurovirulent strain, failed to kill suckling mice after subcutaneous injection, while the less neurovirulent La Crosse virus was also neuroinvasive (see Figure 9.5). After Endres MJ, Valsamakis A, Gonzalez-Scarano F, Nathanson N. Neuroattenuated bunyavirus variant: derivation, characterization and revertant clones. *Journal of Virology* 1990, 64: 1927–1933.

Type 1 poliovirus strain	TCD50 per ml	Enterotropism TCD50 per po ID50	Neurotropism TCD50 per ic PD50
Virulent Mahoney (CNS suspension)	10^6	$10^{3.3}$ (monkeys)	$10^{1.9}$
Attenuated LSc (Tissue culture fluid)	$10^{7.6}$	$\sim 10^4$ (humans)	$>10^{7.6}$

TABLE 9.7 Relative virulence for a target organ can vary widely among virus variants that replicate well in cell culture. A virulent and attenuated type 1 poliovirus are compared, to show that they both replicate well in cell culture (primary monkey kidney cells) and are enterotropic (infectious after oral (po) administration) but differ markedly in their neurovirulence after intracerebral (ic) injection in cynomolgus monkeys
PD50: 50% paralytic dose; ID50: 50% infectious dose. After Sabin AB, Hennessen WA, Winsser J. Studies on variants of poliomyelitis virus. *Journal of Experimental Medicine* 1954, 99: 551–576; Sabin AB. Properties and behavior of orally administered attenuated poliovirus vaccine. *Journal of the American Medical Association* 1957, 164: 1216–1223.

system and this property is distinct from the ability of different variants to invade the target organ from the portal of entry. Figure 9.7 compares four bunyaviruses for their respective pathogenicity for the central nervous system after intracerebral injection.

Attenuated polioviruses offer another illustration of differences in virulence for the target organ that is distinct from peripheral infectivity. In the search for attenuated poliovirus strains for potential use as vaccines, Sabin tested many wildtype and laboratory passaged viruses and found that viruses that replicated well in cell culture (primary monkey kidney fibroblasts) varied widely in their ability to replicate in the CNS of monkeys and chimpanzees. Furthermore, neurovirulence was distinct from enterogenicity, i.e. infectivity after virus feeding (Table 9.7).

Virulence for a target organ may have an indirect explanation. The SARS (severe acute respiratory syndrome) virus is a coronavirus which causes a zoonotic infection of humans, probably after transmission from a bat reservoir through intermediate hosts such as the palm civet. This coronavirus caused an outbreak of human disease in 2002 and 2003, which started in China and then spread to several distant continents (see Chapter 15). Compared to respiratory infections caused by many coronaviruses, SARS had a relatively high mortality (~10%). A possible factor in the virulence of the SARS virus is its use of the ACE2 (angiotensin-converting enzyme 2) molecule as a receptor. ACE2 inactivates angiotensin II, one of the proteins involved in activating the renin-angiotensin system, which can cause several pulmonary edema; ACE2 thus acts a negative regulator of the renin-angiotensin system. It is postulated that the SARS virus binds ACE2, thus releasing a brake on this pro-inflammatory cascade and exacerbating lung edema associated with pulmonary infection by the SARS virus.

Relative pathogenicity for different tissues

Variants of a single virus can differ in their relative pathogenicity for different tissues or organs, which confers a multidimensional character upon virulence. A variant virus may show decreased pathogenicity for one tissue or cell type but enhanced pathogenicity for another cell type. For instance, after intracerebral injection in adult mice, reovirus type 1 Lang strain infects the ependymal lining of the brain and causes hydrocephalus, while reovirus type 3 Dearing strain infects neurons and causes fatal encephalitis. Nevertheless, these two viruses are genetically compatible so that reassortants can be made that include any possible combination of gene segments from type 1 and type 3 viruses. Wildtype mouse hepatitis viruses (MHV, a coronavirus) infect neurons and cause lethal encephalitis upon intracerebral injection of adult mice. Attenuated MHV variants are less infectious for neurons and fail to kill mice acutely, but infect oligodendroglia (cells that produce myelin in the central nervous system) and cause persistent infection and chronic demyelination.

HIV-1 presents another example of differences in tropism. All HIV-1 strains replicate well in primary cultures of peripheral blood mononuclear cells (consisting mainly of T lymphocytes). Some wildtype strains will replicate in primary cultures of monocyte-derived macrophages but not in transformed lines of T lymphocytes. Other HIV-1 strains (including laboratory adapted strains that have been repeatedly passed in T lymphocyte cell lines) will not replicate in macrophages but grow well in T lymphocyte cell lines (Table 9.8). These patterns are explained by the expression of two alternate coreceptors for HIV-1 on different cells and by the restricted ability of most HIV-1 strains to use only one of these two coreceptors, in conjunction with their primary receptor, CD4 (described in Chapter 3). Thus, HIV strains isolated

Viral biotype (Coreceptor usage)	HIV-1 isolate	Growth in each cell type (coreceptor expression)		
		PBMC (CXCR4/CCR5)	Monocyte-derived macrophages (CCR5)	T cell line Sup-T1 (CXCR4)
T-tropic	IIIB	++++	+	++++
(CXCR4)	DV	++++	++	++++
M-tropic	SF162	++++	++++	−
(CCR5)	YU 2	++++	++++	−

TABLE 9.8 Isolates of the same virus can vary in their cellular tropism. All HIV-1 strains replicate well in PBMCs, primary blood mononuclear cells. Laboratory-adapted isolates of HIV-1 are T cell-tropic and do not replicate well in primary macrophage cultures, while wildtype isolates replicate well in macrophages but not in T cell lines. These patterns can be explained by the requirement of HIV-1 strains for different coreceptors that mediate cell entry in conjunction with CD4. Replication was assessed by peak level of p24 antigen production
++++ >100 ng/ml, +++ 10–100 ng/ml, ++ 1–10 ng/ml, + <1 ng/ml, − <0.01 ng/ml. (Macrophages express CXCR4 but at low levels that fail to support the entry of X4 viruses.) Partly after Collman R, Hassan NH, Walker R *et al.* Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1): monocyte- and lymphocyte-tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types. *Journal of Experimental Medicine* 1989, 170: 1149–1163.

Lineage	Virus strain	Neuroinvasiveness (PFU per ip LD50)	Neurovirulence (PFU per ic LD50)
1	USA99b	0.5	0.1
	EGY50	50	0.7
	AUS91	>10 000	3.2
2	SA58	3.2	0.3
	CYP68	>10 000	0.5

TABLE 9.9 Comparative neuroinvasiveness in mice of different strains of West Nile virus (WNV). Virus strains were titrated in 4-week-old NIH Swiss mice by intraperitoneal (ip) or intracerebral (ic) routes of inoculation
After Beasley DWC, Li L, Suderman MT, Barrett ADT. Mouse neuroinvasive phenotype of West Nile virus strains varies depending upon virus genotype. *Virology* 2002, 296: 17–23, with permission.

from the central nervous system are usually macrophage-tropic since the brain has a population of indigenous macrophages (microglia) but normally lacks CD4+ lymphocytes.

West Nile virus (WNV) presents a different example of virulence variation. WNV isolates are grouped into two major lineages, based upon genetic sequence. When tested in mice, there are major variations in neuroinvasiveness within both lineages, although all isolates have high neurovirulence (Table 9.9). West Nile virus was introduced into the USA in 1999 (see Chapter 15) and has spread from New York state across the country to the West coast, with high mortality in wild birds (particularly crows and other corvids) and outbreaks of encephalitis and poliomyelitis in humans. US isolates are among the most neuroinvasive of all known strains of WNV, consistent with the severity of this emerging viral disease. A possible mechanism associated with toll-like receptors is described in Chapter 2.

A different example of qualitative differences between virus variants is the ability of herpes simplex virus (HSV) to be activated from latency. Following natural infection of humans or experimental infection of mice, HSV often establishes latency in sensory ganglia (described in Chapter 10). During latent infection, a single small HSV sequence is actively transcribed, the latency-associated transcript or LAT. HSV variants lacking the LAT sequences can replicate and infect with high efficiency, but have a 'low reactivation' phenotype compared to LAT-positive HSV strains. LAT-positive viruses have the ability to protect infected cells from apoptosis mediated by caspase-8 and this may play a role in activation efficiency.

Host intrinsic response

Animal hosts have evolved a large set of non-immune defenses against viral invaders, many of which have been discovered only recently (see Chapters 4 and 5). In turn, viruses have evolved counter measures to overcome these host defenses and these counter measures are one determinant of virulence. One example is the ability of viral proteins to suppress RNA interference (RNAi) presumably as a counterdefense against this cellular response to many viral infections. It has recently been shown that the

Transfection with protective viral segment (NSs) at 24 hours	Transfection with viral-specific siRNA at 0 hours	Co-transfection with LACV M segment at 0 hours	Expression of LACV G1 protein at 24 hours relative to M segment only
No	No	Yes	100%
No	Yes	Yes	9%
Yes	Yes	Yes	46%

TABLE 9.10 A viral protein can counter the effect of cellular antiviral RNAi. In this example, synthetic small interfering RNAs (siRNAs) were designed to correspond to specific sequences in the genome of La Crosse bunyavirus (LACV). Some of these siRNAs markedly reduced the expression of LACV as measured by the synthesis of LACV G1 glycoprotein in cells transfected with the M segment of LACV. When such cells were pre-treated by transfection with the NSs segment of LACV, the effect of the siRNA was markedly reversed
After Soldan SS, Plassmeyer ML, Matukonis MK, Gonzalez-Scarano F. La Crosse virus nonstructural protein NSs counteracts the effects of short interfering RNA. *Journal of Virology* 2005, 79: 234–244.

NSs protein of La Crosse bunyavirus, the NS1 protein of influenza virus and the E3L protein of vaccinia virus, have the ability to suppress RNAi (Table 9.10).

Host immune response

Altered pathogenicity of variant viruses may be mediated through the host immune response (see Chapter 7). For instance, clone 13 of lymphocytic choriomeningitis virus (LCMV) differed from the Armstrong strain of LCMV from which it was derived by virtue of its ability to replicate more rapidly in dendritic cells and macrophages and less rapidly in the central nervous system. The rapid destruction of dendritic cells interferes with antigen presentation, thereby suppressing the immune response which, in turn, permits the virus to escape clearance. As a result, clone 13 initiates a persistent infection without acute illness, in contrast to Armstrong which produces a benign immunizing infection with rapid virus clearance.

Simian immunodeficiency virus (SIV) produces a high titer viremia and rapidly progressive AIDS, while the Δnef variant produces a modest viremia and a relatively benign infection with occasional AIDS after many years (see Chapter 7). The *nef* gene downregulates the expression of MHC class I molecules on the surface of infected CD4 lymphocytes, protecting them against elimination by antiviral CD8 CTLs and thereby enhancing virus replication. In the absence of the *nef* gene, virus-infected T lymphocytes are more rapidly cleared, enhancing the ability of the host to contain the infection and attenuating the pathogenicity of the virus. In this example, virulence is enhanced by a viral gene that interferes with host immune defenses.

GENETIC DETERMINANTS OF VIRULENCE

Viral virulence, like other viral phenotypes, is encoded in the viral genome and is expressed through the structural and non-structural viral proteins, as well as the non-coding part of the genome. Over the last few decades, a large body of information has been assembled regarding the genetic determinants of virulence. Some of the generalizations that have emerged are summarized in Sidebar 9.1.

SIDEBAR 9.1

Genetic determinants of viral virulence and attenuation

- The use of mutant clones has made it possible to identify the role of specific viral genes and proteins as determinants of virulence.
- There is no 'master' gene or protein that determines virulence and attenuation may be associated with changes in any of the viral proteins as well as the untranslated part of the genome.
- Virulence phenotypes can be altered by very small changes in the genome, if they occur at critical sites. At such critical sites, a change in a single nucleic acid that leads to an alteration in a single amino acid can alter virulence dramatically.
- Variants with mutations in several critical sites may be more attenuated than those with a single point mutation. The frequency of reversion to virulence is inversely proportional to the number of discrete attenuating mutations.
- Reversion to virulence of an attenuated variant can involve back mutation at the genetic site of attenuation, but can also be produced by compensatory mutations at a different site in the same protein or even in another viral protein.
- Attenuating mutations are often host range alterations that affect replication in some cells or tissues but not others.
- Although many attenuated viral variants have been identified, in relatively few instances has the mechanism been identified at a biochemical or structural level.

Reovirus

Reoviruses have a genome consisting of 10 double-stranded RNA segments. Reoviruses fall into three serotypes which possess quite different pathogenic characteristics in mice and yet they reassort readily. Reassortants are prepared by dually infecting a permissive cell culture with reoviruses of two different serotypes and preparing biological clones by plaque purification. Although the gene segments from different reovirus types are interchangeable, the segments from different serotypes can be distinguished by their slightly different sizes that lead to variation in migration rate on polyacrylamide gel electrophoresis, which is used to genotype individual reassortant clones. An example of the use of reassortant viruses is shown in Table 9.11. A number of different pathogenic

Virus	Clone	Origin of gene segment										Pattern of spread
		Outer capsid				Core				NS		
		M2	S1	S4	L2	L1	L3	M1	S2	M3	S3	
Parent	T1L	L	L	L	L	L	L	L	L	L	L	V
	T3D	D	D	D	D	D	D	D	D	D	D	N
Reassortant	R1	D	L	L	L	L	L	L	L	D	D	V
	R2	L	L	L	D	D	D	D	D	L	D	V
	R3	L	L	D	L	L	L	L	L	L	L	V
	R4	D	L	D	D	D	D	D	D	D	D	V
	R5	D	L	L	D	D	D	L	D	D	D	V
	R6	D	L	D	D	D	D	D	D	L	D	V
Reassortant	R7	D	D	D	D	D	D	D	D	D	L	N
	R8	L	D	L	L	D	L	L	L	L	L	N
	R9	L	D	L	L	L	L	L	L	L	L	N
	R10	L	D	L	D	L	L	L	L	L	L	N

TABLE 9.11 The use of reassortant viruses to determine the gene segment (and corresponding viral protein) that encodes a specific pathogenic phenotype. In this example, reovirus Type 1 Lang (T1L) was crossed with reovirus Type 3 Dearing (T3D) to determine the mode of spread to the central nervous system after footpad (subcutaneous) injection of neonatal mice. T3D spread exclusively by the neural route while T1L spread by the hematogenous route (viremia) and the different phenotypes could be determined by sciatic nerve section which prevented T3D from reaching the spinal cord but did not block the spread of T1L. Comparison of a number of reassortant clones shows that the route of spread co-segregates with a single gene segment, the S1 segment that encodes the $\sigma 1$ outer capsid protein
Pattern of spread: V, viremia; N, neural spread. Origin of gene segment: NS, non-structural proteins; L, T1L; D, T3D. After Tyler KL, McPhee DA, Fields BN. Distinct pathways of viral spread in the host determined by reovirus S1 gene segment. *Science* 1986, 233: 770–774.

Organ	Neonatal mice	
	Phenotype	Gene segment
Whole animal	Virulence (lethality)	M2
	Virulence (apoptosis)	S1 ($\sigma 1$s)
	Spread to CNS from periphery	S1
Central nervous system	Neurocellular tropism	S1
Heart	Myocarditis severity	S1, M1, L1, L2
Intestine	Viral titer	S1
	Animal-to-animal spread	L2

TABLE 9.12 An example of viral genes associated with different pathogenic phenotypes. The table shows various reovirus virulence phenotypes that have been mapped to specific gene segments. In some instances, several different genes play a role in the phenotype
After Virgin HV IV, Tyler KL, Dermody TS. Reovirus, chapter 28 in Nathanson N *et al.* (eds), *Viral pathogenesis*, Lippincott Raven Publishers, Philadelphia, 1997.

phenotypes have been described for reovirus infection of mice and many of these have been mapped to specific viral gene segments (Table 9.12). This table also indicates that some of the phenotypes are under multigenic control, an important principle to keep in mind when investigating the viral determinants of virulence.

Bunyavirus

Bunyaviruses are negative or ambisense RNA viruses with a trisegmented genome. The large (L) RNA segment encodes the polymerase (L) protein, the middle-sized (M) encodes two glycoproteins (Gc and Gn; or G1 and G2) and a non-structural protein (NS_m) and the small (S) segment encodes the nucleocapsid (N) protein and a non-structural (NS_s) protein. The California serogroup of the *Bunyavirus* genus of the family Bunyaviridae includes about 12 members that are genetically compatible. Reassortants between these viruses have been used to identify genetic determinants of virulence and attenuation.

Middle (M) RNA segment

Comparison of a wildtype clone of La Crosse virus with an attenuated strain of Tahyna virus (clone 181/57)

Relative virulence	Virus Clone	Score	Nucleotide									
			220	472	871	2034	3333	3464	4064	6127	7165	7432
More virulent	P3/L	2.71										
	SV1/L	2.68					A					
	SP2/L	2.51						A				
	S3′/L	2.40								A	A	A
	SLR2	2.39					A	A	A	A	A	A
Intermediate	L472V3/S	2.07	A		A		A	A	A	A	A	A
	SCC/L	1.93			A	A	A	A				
	SV3/L	1.74				A						
	L472/S	1.58	A		A	A	A	A	A	A	A	A
	LV3/S	1.32	A	A	A		A	A	A	A	A	A
	ST/L	1.14	A	A	A							
Less virulent	P3/S	0.41	A	A	A	A	A	A	A	A	A	A
	SLR1	0.28	A	A	A	A						

TABLE 9.13 Analysis of genetic determinants of virulence at the nucleotide level. This example compares wildtype virulent type 3 poliovirus (P3/Leon) with the attenuated type 3 OPV (P3/Sabin) derived therefrom. There are 10 nucleotide differences between the two viruses (in the whole genome of >7000 nucleotides) and a set of recombinant viruses were constructed to determine which divergent nucleotides influenced virulence. In this instance, it appeared that nucleotides 220 and 472 in the 5′ non-translated region and nucleotides 871 (VP4) and 2034 (amino acid 91 in VP3) were the most significant critical sites. Red boxes indicate that the 5 most virulent recombinant viruses had the 'virulent' nucleotide at all 4 of these sites while the two most attenuated recombinant viruses had the 'attenuated' nucleotide at these 4 sites

Virus clone: P3/L, virulent parental P3 Leon strain; P3/S, attenuated P3 Sabin strain; Score: neurovirulence score based on severity of spinal cord lesions after intracerebral injection of macaques; A, nucleotides found in the attenuated but not the virulent virus. After Minor PD. The molecular biology of poliovaccines. *Journal of General Virology* 1992, 73: 3065–3077.

documented that the attenuated virus was highly neurovirulent in mice but was not neuroinvasive after subcutaneous injection of suckling mice (see Figure 9.5). Studies of reassortants between the two viruses showed that neuroinvasiveness co-segregated with the M RNA segment.

Large (L) RNA segment

Comparison of a neuroattenuated clone (B.5) derived from a reassortant of La Crosse and Tahyna virus showed that the attenuated variant had reduced ability to replicate in neuroblastoma cell cultures and reduced ability to replicate after intracerebral injection in adult mice (see Figure 9.3). Reassortants between the attenuated virus and a neurovirulent virus showed that neurovirulence co-segregated with the L RNA segment (see Figure 9.2).

Poliovirus

Polioviruses are single-stranded RNA viruses of positive polarity (their genome can act as mRNA and be translated directly into proteins) and cDNA clones constructed from the viral RNA can be used for manipulation of the genome. The altered cDNA clones are infectious, i.e. they can be transfected into permissive cells with the production of infectious virus. These features have facilitated the mapping of poliovirus virulence.

Oral poliovirus vaccine (OPV) is comprised of attenuated clones of each of the three poliovirus serotypes which, compared to virulent wildtype polioviruses, have markedly reduced neurovirulence on direct intrathalamic or intraspinal injection in macaques (see Table 9.7). There are a number of differences between the OPV strains and their wildtype parents, acquired during many successive passages in cell culture and only a subset of these are relevant to the neurovirulent phenotype. More specific information can be obtained from OPV viruses that have reverted to virulence after feeding to humans, which is a common consequence of replication in the human GI tract. Such virulent revertants have a smaller number of nucleotide differences from the attenuated vaccine strains than do the parent wildtype viruses. Finally, engineered recombinant viruses have been used deliberately to test the influence of individual critical nucleotides (Table 9.13).

For all three of the attenuated OPV strains, there are two sets of neurovirulence determinants. For each strain, there is a critical nucleotide in the 5′ non-translated region of the genome, at positions, 480, 481 or 472, for types 1, 2 and 3, respectively. Based on computer modelling of their secondary structure, the 5′ RNA is predicted to form stem loops at positions 470 to 540. This region is thought to be involved with initiation of translation at

Viral gene	Genetic composition of parent and reassortant viruses			
HA	1918	Tx/91	1918	Tx/91
NA	1918	1918	1918	Tx/91
M	1918	1918	1918	Tx/91
NP	1918	1918	1918	Tx/91
NS	1918	1918	1918	Tx/91
P1	1918	1918	Tx/91	Tx/91
P2	1918	1918	Tx/91	Tx/91
P3	1918	1918	Tx/91	Tx/91
Titer in lung (Log_{10} EID50 per ml)	7.5	5.2	6.0	3.0
Mortality in mice (10^6 PFU intranasal)	100%	0%	100%	0%
Survival (days)	3		6.2	

TABLE 9.14 Multigenic determination of virulence. The 1918 influenza virus (H1N1 antigenic type) was reconstructed and used to make reassortants with a contemporary human H1N1 isolate (Tx/91). Parent and reassortant viruses were tested for virulence after intranasal inoculation of adult mice. Reassortants encoding many of the genes of the virulent 1918 virus showed considerable virulence but were less lethal than the parent 1918 virus, indicating that virulence was associated with the full spectrum of viral genes acting in concert

After Tumpey TM, Basler CF, Aguilar PV *et al.* Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* 2005, 310: 77–80.

an internal ribosomal entry site (IRES) and mutations at the critical site are predicted to perturb the secondary structure and alter the initiation of translation. However, a recent study failed to support the role of the IRES in determining the attenuation of type 3 OPV or in explaining the tissue tropism of poliovirus.

The second groups of critical sites involves the structural (VP1 to VP4) and non-structural viral proteins, but is disparate for the three attenuated strains of OPV. For type 1, point mutations associated with virulence are located in several capsid proteins and the non-structural polymerase; for type 2 OPV, mutations are in VP1; and for type 3 OPV, mutations in VP3 or VP4 are involved. It is not clear how mutations in the structural proteins alter virulence, but it has been hypothesized that these are implicated in viral uncoating or in viral assembly.

OPV strains are temperature sensitive and represent host range mutants, since they replicate well in primate fibroblasts but poorly in neuroblastoma cells, compared to their virulent counterparts. Thus, it must be presumed that viral determinants of uncoating, translation or assembly are cell specific and must ultimately be explained in terms of the subtle variations in the activity of poliovirus proteins in various cell types.

Influenza virus

Influenza is an eight-segmented negative strand RNA virus that causes acute infection of the lower respiratory tract in mammals. Different strains vary in virulence, depending on at least two aspects of pathogenesis, severity of pulmonary disease and ability to spread systemically. The virulence of avian influenza viruses for birds has usually been associated with its ability to spread systemically which, in turn, is determined by the susceptibility of the viral hemagglutinin to be cleaved by cellular proteases. The hemagglutinin is translated as a precursor molecule that must be cleaved to two peptides (HA1 and HA2) in order for the mature virus to complete entry into susceptible cells. Virulent avian strains can be cleaved in the endoplasmic reticulum by furin, while avirulent strains depend on cleavage by extracellular proteases (see Chapters 4 and 15). Intracellular cleavage confers the ability to spread systemically, which is associated with high virulence in avian hosts.

The 1918 influenza outbreak was a notable global pandemic that is estimated to have caused up to 50 million deaths. Since this outbreak occurred prior to the introduction of methods for the isolation of influenza viruses (1933), there are no contemporaneous isolates available and the virulence of this virus has been a matter for speculation. An extraordinary effort in viral archaeology, using RT-PCR to recover viral fragments from pathological and frozen specimens, has gradually revealed the sequence of the 1918 virus. In 2005, the sequence was completed and reverse genetics was used to reconstruct the whole virus. The reconstructed virus is highly virulent in mice and reveals several interesting features. The 1918 virus is an H1N1 virus and all eight segments appear to have been derived from avian (rather than human) influenza viruses. Virulence in this instance is associated with very severe lung infection and not with systemic spread. In contrast with avian influenza viruses, virulence is highly multigenic and is not specifically associated with cleavability of the hemagglutinin (Table 9.14).

In the mid-1990s, a virulent H5N1 variant of avian influenza virus appeared in southeast Asia, where it has caused many severe outbreaks in domestic poultry, requiring large scale culling programs for containment. In 2006, this virus caused outbreaks in wild waterfowl and spread from Asia to Europe. Cumulatively, through Spring, 2006, the avian H5N1 virus has infected about 200 humans who had contact with poultry. The mortality rate in humans has been ~50%, raising serious concerns of a human pandemic similar to the 1918 epidemic. However, there has been no evidence of human-to-human spread of this virus. Recent genetic analyses have suggested that there are at least three virulence determinants in this H5N1 virus: basic amino acids adjacent to the cleavage site in the viral hemagglutinin; amino acid 627 in PB2, one of the viral polymerase molecules; and a short sequence in the NS1 protein that may influence interferon or other host response.

VIRULENCE GENES OF CELLULAR ORIGIN

In the last decade, a new class of virus-encoded proteins has been recognized that contribute to virulence of viruses by mimicking cellular proteins. It is hypothesized that viruses have derived the genes that encode these proteins from the cells in which they replicate, by recombination and subsequent modification. Most of these cell-derived genes have been identified in DNA viruses with large genomes, such as the herpes and poxviruses, which have a greater capacity to accommodate accessory genetic information than do viruses with small genomes. From an evolutionary viewpoint, the cell-derived genes resemble oncogenes but, rather than endowing the virus with transforming properties, they act to subvert the antiviral defenses of the infected host.

Cell-derived viral genes, which include 'virokines' and 'viroceptors', enhance virulence through many different mechanisms. Virokines secreted from infected cells can mimic the action of cytokines, causing host cells to proliferate, thereby increasing virus production. Viroceptors resemble receptors for cytokines and can act as decoys, binding and sequestering cellular cytokines. Some virus-encoded proteins bind antibodies or complement components, reducing the lysis of virus-infected cells, while others perturb antigen presentation or immune induction. A few selected examples are described below.

Poxviruses

Vaccinia complement control protein (VCP)

The complement cascade, described in Chapter 5, consists of a group of proteins that produce a membrane attack complex that can destroy microbial pathogens or virus-infected cells. Poxvirus infections induce antiviral antibodies that can trigger the complement cascade and such antibodies destroy virus-infected cells, thereby acting as a host defense. Vaccinia, a poxvirus, encodes VCP (vaccinia complement control protein), a protein that abrogates the complement-mediated attack on virus-infected cells. VCP resembles a human plasma protein, C4-BP, that monitors the complement cascade and prevent its unwanted activation, by binding to C4b, one of the intermediaries in the cascade. VCP also binds C4b, thereby blocking the complement cascade and vitiating complement-mediated host defenses. VCP-minus mutants are less pathogenic in vivo than wildtype vaccinia virus, illustrating the in vivo effect of VCP (see Chapter 8).

Tumor necrosis factor (TNF) viroceptors

TNF is a family of pro-inflammatory cytokines that are produced by activated macrophages and T lymphocytes. TNFs act by binding to cognate receptors that are expressed on myeloid and lymphoid cells, producing multiple effects on immune networks and host responses to infection. A number of poxviruses encode a viroceptor that is a soluble homolog of the cellular p75TNF receptor. It is presumed that the soluble receptor binds and sequesters TNF, thus modulating TNF-mediated cellular responses to infection. Poxviruses encode a large number of soluble proteins, some of which have been shown to act as receptors for other cytokines such as interferon γ, interferon α/β and interleukin 1β.

Interleukin 4 (IL-4)

During immune induction, IL-4 is produced mainly by the T_H2 subset of CD4+ helper cells and downregulates T_H1 activation, deviating the immune response towards B lymphocytes and away from the induction of CD8+ CTLs. When mousepox virus is engineered to express IL-4, the virus becomes much more virulent, associated with a reduced induction of virus-specific CTLs and also a reduced induction of IFNγ. Although an experimental artefact, this demonstrates the potential consequence of incorporation of a cellular gene into a viral genome.

Toll-like receptors (TLRs)

The A46R protein of vaccinia virus blocks the host innate immune response. TLRs initiate an intracellular cascade that leads to the downstream activation of NFκB (see Chapter 5). A46R binds to myeloid differentiation factor 88 (MyD88) and several other proteins that participate in intracellular signaling, thereby reducing the innate immune response. A vaccinia virus mutant lacking A46R was less virulent, compared to wildtype virus, in an intranasal infection model in mice.

Herpesviruses

Herpes simplex virus (HSV) Fc receptors (FcR)

Two of the glycoproteins of HSV, gE and gI, act together as a receptor for the Fc domain of immunoglobulins. HSV infection induces the host to produce antiviral antibodies that can initiate the lysis of HSV-infected cells by cell-mediated cytotoxicity (ADCC). HSV counters this host defense by the action of gE and gI. When antiviral antibodies bind via their Fab domains to virus-infected cells, their Fc domain is bound to gE/gI and cannot trigger ADCC (see Chapter 8). This protects virus-infected cells from lytic attack, thereby prolonging their production of virus. Cells infected with a gE-deleted mutant HSV are much more sensitive to ADCC than are cells infected with wildtype HSV.

Marek's disease virus (MDV), an α-herpesvirus that causes an acutely fatal lymphoproliferative disease in poultry, has a gene (vIL-8), which encodes a protein that resembles IL-8, a cytokine that attracts and mobilizes neutrophils and T lymphocytes. An MDV variant in which vIL-8 has been deleted, is much attenuated and re-introduction of the gene restores virulence (Table 9.15). Presumably vIL-8 acts as a decoy and subverts the function of cellular IL-8, thereby impairing a host defense mechanism.

REPRISE

Virulence denotes the relative ability of viral variants to cause disease and can be measured in a variety of ways,

Strain of MDV	Transient paralysis	Gross visceral lesions	Total mortality
rMd5	12/17	10/17	15/17
rMd5-ΔvIL-8	0/17	2/17	1/17
rMd5-ΔvIL-8-RV	11/17	11/17	17/17

TABLE 9.15 An accessory gene of herpesvirus that is a cellular homolog enhances viral virulence. Disease manifestations in chickens inoculated with a virulent strain of Marek's disease virus (MDV), a variant in which the vIL-8 gene has been deleted and a revertant that expresses vIL-8

After Cui X, Lee LF, Hunt HD, Reed WM, Lupiani B, Reddy SM. A Marek's disease virus vIL-8 deletion mutant has attenuated virulence and confers protection against challenge with a very virulent plus strain. *Avian Diseases* 2005, 49: 199–206, with permission.

based on illness, death or pathological lesions, each of which can be quantified. Relative virulence is a complex phenotype, since it depends upon variables such as the species and age of host, the route of infection and the dose of virus. The virulence phenotype also may be qualitative, involving differences in the tropism of different viral variants. Wildtype isolates of a virus may vary in virulence and virulence variants can also be selected by experimental manipulation. Attenuated variants often exhibit host range or temperature sensitive phenotypes and may provide candidates for live virus vaccines. Virulence phenotype can be manifest at any step during the course of infection, from invasion, to spread, involvement of target organs or shedding. Virulence or attenuation can be mapped to specific viral genes and individual nucleotides and may be associated with any viral genes, including non-coding sequences. In some instances, virulence is associated with individual viral genes and, in other cases, is polygenic. For viruses with large genomes, virulence may be conferred by accessory genes that act as virokines or viroceptors, some of which appear to have originated from eukaryotic genes.

FURTHER READING

Reviews, chapters and books

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Chapter 10

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Viral Persistence

Neal Nathanson and Francisco Gonzalez-Scarano

INTRODUCTION

The prototypical viral infections are acute and induce host defensive responses that clear the foreign agent and leave the host with long-lasting virus-specific immunity. However, a significant number of viruses are capable of persisting, often for the lifetime of the host. In order to persist, a delicate balance must be achieved so that, on the one hand, the host is not killed by the destructive effects of the virus while, on the other hand, the virus is able to evade the multitude of immune defenses that act to eliminate it. How this happens is the theme of this chapter.

The mechanisms of persistence range along a spectrum (Sidebar 10.1). At one extreme are viruses that continue to *replicate at high titers* over long periods of time, while at the other extreme are viruses that become *latent*, emerging at rare intervals to replicate for short periods of time.

SIDEBAR 10.1

Rules of persistence in vivo

- RNA and DNA viruses from many different families can persist in vivo.
- Strategies for persistence vary widely, from full blown 'high titer' replication to 'latency', with intermediate examples of 'smoldering' infection.
- For each viral strategy, there is a corresponding strategy for evasion of host immune surveillance.
- High titer replication requires that virus either be non-cytocidal or that there is rapid replacement by cellular proliferation of target cells. Immune surveillance is unable to eliminate the virus, due to tolerance, immune complex formation, viral mutation, or other mechanisms.
- Latency usually requires that the viral genome can persist in a non-replicating mode, either integrated into the genome of the host cell or as an episome, although intermittent active replication may occur. Immune surveillance may be competent to eliminate the virus, which can only recur for brief intervals, or recurrence may be associated with intermittent reduction of immune defenses.
- Smoldering infections involve continuous productive infection and cell-to-cell transmission at a low level. Potentially effective immune surveillance is circumvented by mechanisms such as antigenic variation, infectious immune complexes, or intercellular bridges.

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Virus family Example	Host(s)	Site of persistence	Cytocidal in permissive cells	Immune response
High titer replication				
Arenaviridae LCMV	Mouse	Macrophage	No	Restricted
Hepadnaviridae HBV	Human	Hepatocyte	No	Restricted
Latent infection				
Herpesviridae HSV	Human	Sensory neuron	Yes	Brisk
Polyomaviridae Papilloma	Human	Epidermal cells	Yes	Brisk
Smoldering infection				
Picornaviridae TMEV	Mouse	CNS Glial cells	Yes	Normal
Paramyxoviridae Measles	Human	Neurons	Yes	Super normal
Lentiviridae HIV	Human	CD4 lymphocyte	Yes	Variable

TABLE 10.1 A selected list of human and animal virus that employ different styles of persistence

Between both ends of the spectrum are examples of *smoldering infections* that share characteristics of both replication and latency. Viruses employ a variety of strategies to escape immune surveillance and these tend to be specific for different styles of persistence. Thus, immune tolerance often characterizes high titer persistent infections, while active immune responses are seen in many latent infections. A few selected examples of each style of persistence are listed in Table 10.1. The viruses selected for discussion represent some of the best studied examples, but the mechanisms of persistence are less well analyzed or more complex in many uncited instances.

For some viruses, persistence may be partially determined by the conditions of infection. For instance, lymphocytic choriomeningitis virus (LCMV) causes a persistent infection in newborn mice but not in adult mice, as is true of several viruses of humans such as hepatitis B virus (see Chapter 7). On the other hand, numerous herpes viruses cause persistent infections regardless of age at infection.

CELL CULTURE MODELS OF VIRAL PERSISTENCE

Many viruses can establish persistent infections in cultured mammalian cells. Although artificial, these models illustrate certain principles of virus–cell interaction in the absence of the complexities associated with infection of animals. Persistently infected cell cultures (sometimes called 'carrier cultures') fall into two categories, those involving non-cytocidal viruses and those involving lytic viruses (Table 10.2).

Non-lytic viruses

There are several hallmarks of carrier cultures produced by non-lytic viruses. All cells in the culture are infected and all daughter cultures established by the cloning of single cells are also infected. Conversely, it is usually impossible to 'cure' the cultures by antiviral treatments, such as interferon or virus-specific antibodies. During extended passaging, free infectious virus may wane or even disappear, due to evolution from a replication competent virus to defective genomes that may or may not produce budding viral particles. The continued presence of viral genomes can be detected by the presence of intracellular viral antigens or viral genetic sequences, or by the resistance of carrier cultures to superinfection with the same virus.

Characteristics of persistently infected cell culture	Non-lytic virus	Lytic virus
What fraction of the cells are infected?	~100%	<100%
Are single cell clones always infected?	Yes	No
Must antiviral factors be present in the culture medium to protect the cells?	No	Yes (1)
Can the culture be 'cured' by adding antiviral antibody or interferon?	No	Yes (1)
Does the culture resist superinfection with the same virus?	Yes (1)	No (2)

TABLE 10.2 Characteristics of lytic and non-lytic persistent viral infection of cell cultures. These generalities apply to many but not all instances
(1) Usual finding but there are exceptions. (2) Depends on experimental conditions and method of assay. After Walker DL. The viral carrier state in animal cell cultures. *Progress in Medical Virology* 1964, 6: 111–148, with permission.

Lytic viruses

Surprisingly, lytic viruses can often establish carrier cultures, although not as readily as non-lytic viruses.

Characteristically, such carrier cultures are composed of a mix of infected and uninfected cells. Persistence depends on an equilibrium between the loss of infected cells and the increase of uninfected cells by cell division. Often, this equilibrium requires the presence of antiviral agents, such as interferon, which act as governors on virus replication. If this antiviral activity is removed by frequent changes of the culture medium, the culture may be destroyed. Conversely, such cultures can be 'cured' by the addition of antiviral agents such as specific antibody. If single cell clones are derived from such carrier cultures, some of them will usually give rise to uninfected cultures.

Variation in virus and cells

Continuous passage of carrier cultures may result in evolution of either virus or cells. As mentioned above, the infecting virus may be replaced by defective virus or by replication competent virus with reduced cytopathogenicity. Carrier cultures established by lytic viruses may lead to the selection of cells that are relatively resistant to destruction by the virus. Likely, the artificial conditions of persistent infection select for pre-existing viral or cellular variants that have a survival advantage in carrier cultures. Virus variants may exhibit reduced pathogenicity due to reduced efficiency at any of the steps in the replication cycle or due to diminished triggering of apoptosis. Cellular variants may be resistant because of differences in any of the multiple cellular molecules that are involved in the steps from virus entry to release.

Using genetic reassortants, it is possible to map the variant viral genes and proteins selected during continuous passage. Reovirus is usually lytic in L929 cells (a continuous murine cell line) but carrier cultures can be established under controlled conditions. Virus isolated from these cultures is less lytic than wildtype virus and changes in the S4 and S1 genes, that encode the σ3 and σ1 proteins, are responsible for establishing and maintaining the persistent phenotype (Table 10.3). The σ3 protein is the major outer capsid protein and the σ1 protein is the virus attachment protein. It appears that the variant capsid proteins associated with persistent viral variants reduce the efficiency of viral entry and the likelihood of overwhelming lytic infection, thus promoting the establishment of persistence.

Certain aspects of cell culture models are relevant to persistence in animals. Studies with non-lytic viruses demonstrate that long-term infections can readily be established, that they spread widely through the population of susceptible cells and that variant viruses, often with reduced rates of replication, may be selected during long-term persistence. Observations on lytic viruses indicate that, surprisingly, they can often persist in a population of susceptible cells, but that this usually requires an extraneous antiviral modulator that prevents total destruction of the cell population. Furthermore, during persistence there is a tendency for evolution of a less lytic virus–cell relationship, both through selection of variant viruses that are less destructive and the selection of cells that are less permissive for virus replication. These observations foretell some of the characteristic features of viral persistence in animal hosts.

Gene segments	Predominant gene segments (Type 2wt or Type 3ts)	
	Day 16	Day 230
L1	2	2
L2	2/3	2
L3	2	2
M1	2	2
M2	2	2
M3	2	2
S1	3/2	3
S2	2	2
S3	2	2
S4	3/2	3

TABLE 10.3 Genetic determinants of viral persistence in cell culture. Reovirus is a lytic virus but can be induced to cause persistent infections in cell culture by co-infecting cultures with a lytic wildtype virus (Type 2 wt) and a temperature sensitive variant (Type 3 ts). The virus isolated late after persistent infection is a reassortant carrying the genes of the T2 wt virus except for the S4 and S1 genes of the T3 ts variant virus and it appears that these two gene segments are responsible for the persistent phenotype
After Ahmed R, Fields BN. Role of the S4 gene in the establishment of persistent reovirus infections in L cells. *Cell* 1982, 28: 605–612, with permission.

IMMUNE CLEARANCE OF ACUTE VIRAL INFECTION

As a prelude to consideration of persistence, it is useful to recapitulate briefly the mechanisms by which the immune response controls and eliminates an acute virus infection (see Chapter 6). Effector T lymphocytes can destroy virus-infected cells, produce antiviral cytokines and recruit mononuclear cells to sites of viral replication and destruction, while antibody neutralizes and opsonizes free infectious virions. In some instances, both antibody and virus-specific effector lymphocytes can purge virus-infected cells without destroying them. It is these mechanisms that a virus must evade in order to persist.

An example where viral clearance appears to be mediated by antibody is shown in Figure 2.5, which illustrates the disappearance of poliovirus from the plasma concomitant with the appearance of serum neutralizing antibody. Evidence for the role of the immune response in the elimination of virus from solid tissues is based on the effect of experimental immunosuppression. Figure 10.1 summarizes an experiment in which an immunosuppressive drug abrogates the clearance of a neurotropic virus from the brain, indicating the ability of intact host immune defenses to terminate a viral infection and eliminate the causal agent.

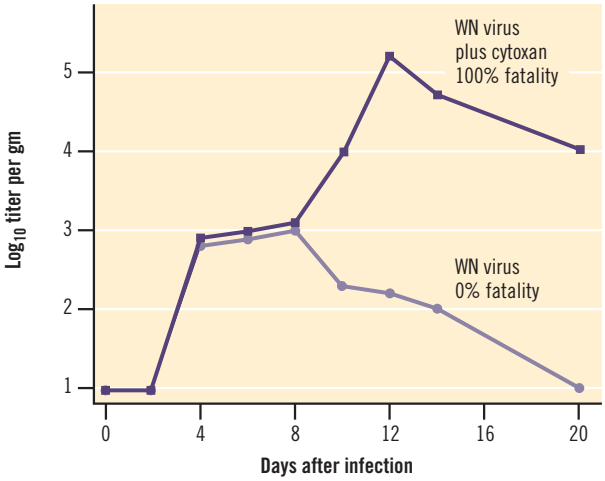

FIGURE 10.1 Enhancement of an acute infection by chemical immunosuppression to implicate the role of the immune response in termination of an acute viral infection. Adult rats were injected intracerebrally with $10^{6.3}$ suckling mouse LD50 of West Nile virus (WNV), a togavirus and one group was immunosuppressed by three doses of cyclophosphamide on days 1, 8 and 14 after infection. In normal rats, WNV causes a minimal non-lethal infection of brain and the virus is completely cleared by 20 days after infection. Immunosuppression potentiates the infection, which is not cleared but spreads slowly to kill all animals by 20 days. The curves indicate that suppression does not alter the replication of the virus for the first week of infection but interferes with the clearance process thereafter. After Cole GA, Nathanson N. Potentiation of experimental arbovirus encephalitis by immunosuppressive doses of cyclophosphamide. *Nature* 1968, 220: 399–401, with permission.

MECHANISMS OF PERSISTENCE AND ESCAPE FROM IMMUNE SURVEILLANCE

Three patterns of persistence are diagrammed in Figure 10.2 and are contrasted with an acute infection. If the length of an acute virus infection is defined as the period from acquisition of infection to total elimination, then the duration of an acute infection varies from about one week (for rhinoviruses) to ~6 months (for hepatitis B virus). Beyond those limits infections may be considered persistent. Some persistent infections undergo spontaneous termination, so that the duration of persistence in individual hosts varies from months to lifelong. This variability reflects the delicate balance between parasite and host. (Rabies virus, a virus that produces acute infections with incubation periods ranging up to months or years, is a special exception.)

High titer persistent infections and immune tolerance

For a persistent virus to replicate at high titer, it must avoid catastrophic pathogenic effects, either because it is not acutely cytocidal or because it attacks target cells that can be replenished by a very high rate of proliferation. Many viruses can replicate productively without causing cell death (see Chapter 4) and a number of them can cause persistent infections. In such instances, the initial dynamics resemble those of an acute infection, following which the virus titer decreases somewhat but then reaches a setpoint that may be maintained indefinitely

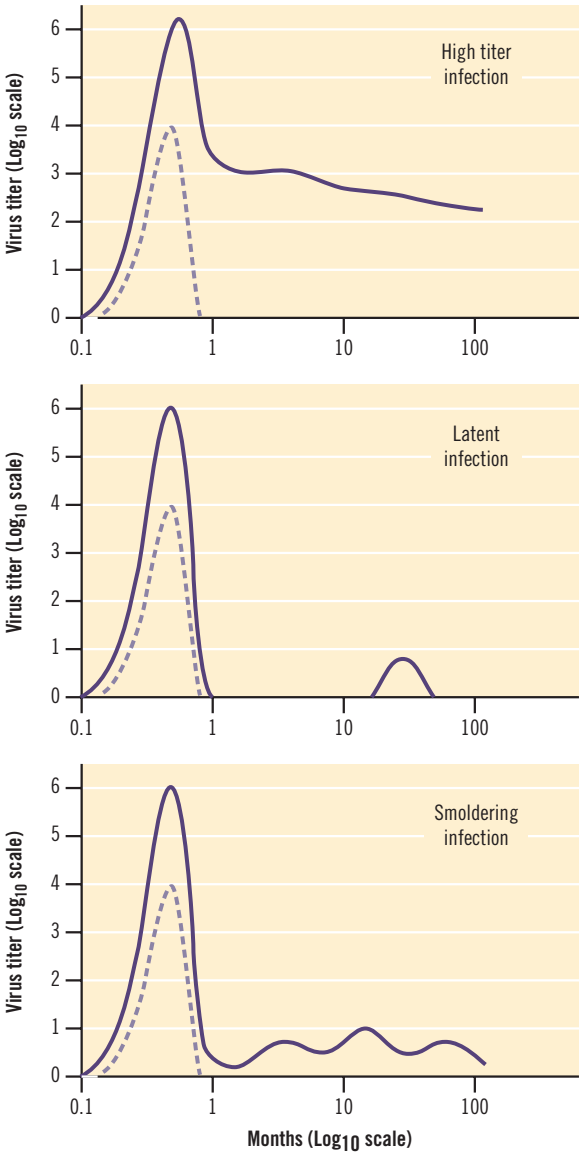

FIGURE 10.2 Patterns of virus persistence. For reference, an acute infection is shown as a dashed line in all panels. Upper panel: high titer infection. An acute phase is often apparent, following which the titer drops but persists at a high level for a long time, during which virus titers may remain stable or gradually wane. Middle panel: latent infection. An acute infection is followed by disappearance of replicating virus which persists only as a latent genome. Periodic recrudescence occurs that may be accompanied by signs of disease. Intervals between recrudescences may last from weeks to many years and some infected individuals may never experience a recrudescence. Bottom panel: smoldering infection. An acute infection of varying extent is followed by marked reduction in overt virus replication but infectious virus may be frequently recovered indicating that true latency has not occurred. Modified after Johnson R. *Neurotropic virus diseases*, Raven Press, 1985, with permission.

or gradually decline. Examples of this pattern are hepatitis B virus (HBV) (Chapter 7), lymphocytic choriomeningitis virus (LCMV) (Chapter 8) and human immunodeficiency virus (HIV) (Chapter 14).

Non-lytic viruses

High titer persistence is often characterized by 'tolerance', an apparent absence of virus-specific immunity. The

mechanisms by which the tolerance can be induced include deletion of 'forbidden' clones of naïve T lymphocytes in the thymus, or exhaustion of peripheral virus-specific T lymphocytes in the presence of excess antigen (discussed in Chapter 8). Tolerance may be limited to specific components of the effector limb of the immune response. For instance, hepatitis B persistence is characterized by absence of antiviral antibody against HbsAg, while anti-HBcAg antibody may be induced. LCMV persistence is characterized by absence of cellular immune responses while virus-specific antibody is produced.

Defects in the immunobiological potency of memory T cells can also induce the tolerance phenotype. Recent studies with LCMV have shown a marked difference in the properties of CD8+ virus-specific memory T cells that are generated during acute versus persistent infections. Different clones of LCMV were used to produce acute or persistent infections. The acute infection is fully cleared within two weeks while the persistent infection is controlled after two months with lifelong residual virus in some tissues. When a comparison is made of mice at three months after infection, viral epitope-specific memory CD8+ T cells were present that were maintained for at least one year after infection. However, acute infections induced memory cells that exhibited the cardinal properties of self-renewal in the absence of antigen and high levels of receptors for IL-7 and IL-15, while the memory cells isolated from animals with persistent infections expressed low levels of these two interleukin receptors and gradually disappeared in the absence of antigen (Figure 10.3). In this model, the memory cells associated with persistent infection may be less capable of differentiating into effector CD8+ T cells capable of eradicating persistent infection.

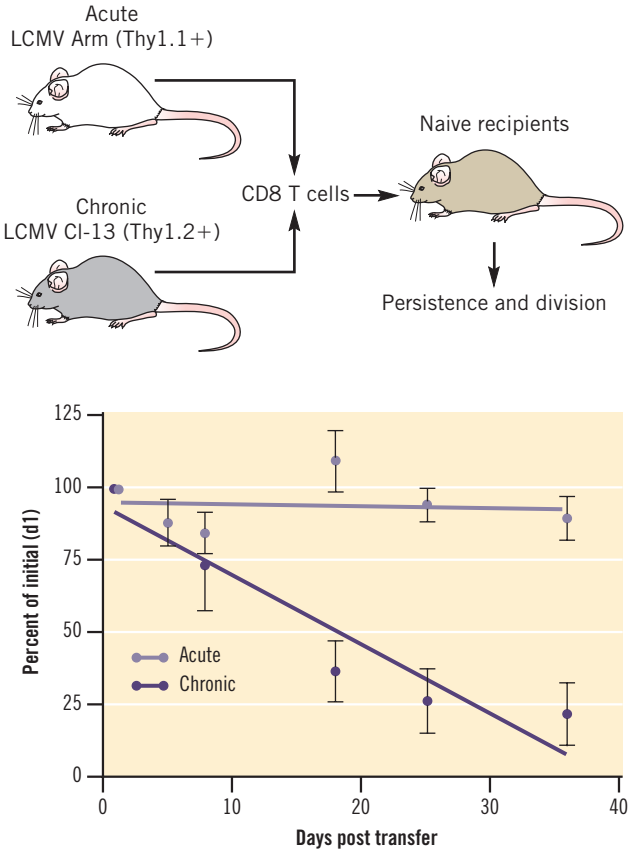

FIGURE 10.3 Virus specific memory CD8+ T cells induced by persistent infections do not persist in the absence of antigen. Top: two strains of LCMV were used to produce acute or persistent infections of mice, using syngeneic mice that were either Thy1.1 or Thy 1.2 positive (Thy1.X is an allelic marker on T cells that does not cause histoincompatibility). In acute infections the virus was completely cleared within 1–2 weeks, while in persistent infections, the virus was reduced to low levels by 2–3 months, but was never completely eliminated. Spleen cells obtained at 3 months from both groups of mice were tested for survival of virus-specific memory CD8+ T cells by transfer into uninfected syngeneic recipients. Bottom: cells from both groups of mice (separated by the Thy marker) were tested for their ELISPOT responses when stimulated by an immunodominant LCMV peptide, as a measure of the ability of memory T cells to generate antigen-responding effector T cells. After Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proceedings of the National Academy of Sciences* 2004, 101: 16004–16009.

Lymphocytic choriomeningitis virus (LCMV)

Evidence for the role of immune tolerance in maintaining viral persistence is provided by the experimental termination of persistence by intravenous injection of virus-specific CD8 cells. Figure 10.4, summarizing an experiment with LCMV, exemplifies this phenomenon. Similar results have been obtained with HBV, where CTLs specific for HBs epitopes cleared virus from hepatocytes (see Chapter 6).

It was noted that the expression of the viral genome may evolve in carrier cultures and the same phenomenon has been observed in vivo. For instance, following the infection of newborn mice with LCMV, the nucleoprotein and envelope glycoproteins are both expressed in infected neurons for the first week, but over the subsequent 10 weeks the expression of glycoproteins gradually wanes while there is no diminution in nucleoprotein levels. This phenomenon may play a role in persistence, since the absence of glycoproteins (the only viral protein expressed on the cell surface) would make neurons poor targets for antibody recognition.

Friend virus

A different mechanism of 'tolerance' appears to be operative in persistent infection with Friend virus, a complex of several retroviruses that causes persistent infection and lethal leukemia in most strains of inbred mice. Control of this virus during acute infection is mediated mainly by CD8+ T cells, but adoptive transfer of CD8+ cells from persistently infected mice indicates that they lack the ability to clear infection. CD8+ cells from acutely infected mice produced robust levels of three effector molecules, perforin, granzyme A and granzyme B, but CD8+ cells from persistently infected mice were deficient in cytolyic activity. Further investigation revealed that this reduced efficacy was due to a modulatory effect of CD4+ T cells; when the transferred cells were treated with a monoclonal antibody (anti-GITR) known to reduce the potency of regulatory CD4+ T cells, CD8+ cells regained their efficacy. In this instance, persistence is associated with the activity of regulatory CD4+

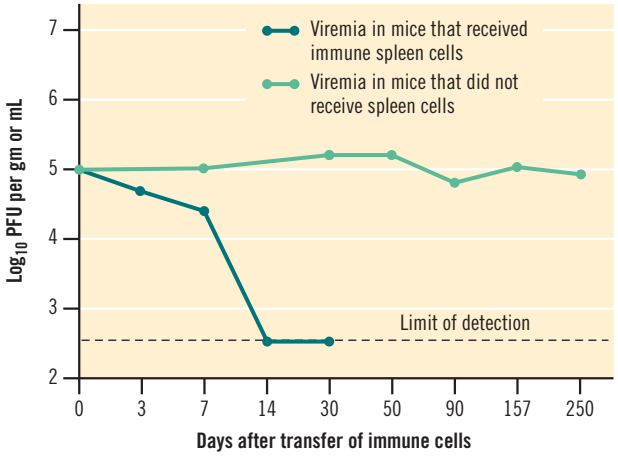

FIGURE 10.4 Immune T lymphocytes can clear a persistent virus infection, implying that (in some instances) persistence involves escape from the cellular arm of the immune response. In this example, LCMV causes persistent life-long infection of mice. When such mice are treated by adoptive immunization of CTLs from an immune donor ($10^{7.3}$ spleen cells from adult mice immunized by infection 60 days prior to transfer), the virus is cleared. The specificity of the process is shown by the requirement for syngeneic donor lymphocytes and by the ability of clones of CD8 T lymphocytes specific for an individual viral epitope to clear infection. The rate of clearance differs for different organs and tissues, implying some differences in the action of effector cells and in the mechanisms of immune evasion. After Ahmed R, Jamieson B, Porter DD. Immune therapy of a persistent and disseminated viral infection. *Journal of Virology* 1987, 61: 3920–3929, with permission.

T cells that inhibit the effector function of virus-specific CD8+ CTLs.

Lytic viruses

It is unusual for high titer persistence to be produced by a cytolytic virus, but the primate lentiviruses represent an important exception. Figure 8.11 shows the persistent viremia produced by a pathogenic strain of simian immunodeficiency virus (SIV). The main target cells for these lentiviruses are CD4 lymphocytes that undergo lytic infection. It has been calculated that the continuous destruction of CD4 cells results in a reduction of the average half-life of these cells from 75 to 25 days. However, the bone marrow is able to respond to the abnormal rate of destruction by increasing the production of naïve CD4 cells, at a rate sufficient to maintain a reasonable concentration of circulating CD4 cells. This permits a lytic virus to persist at a high titer for an extended period of time in the relative absence of clinical illness, although eventually the bone marrow is unable to compensate and CD4 levels drop, leading to functional immunodeficiency. The pathogenesis of HIV and AIDS is discussed in Chapter 14.

In contrast to most high titer persistent infections, lentiviruses induce immune responses rather than tolerance. The immune response to lentiviruses is quite effective, as judged by its ability rapidly to contain the acute phase of infection, resulting in a reduction from peak viremia at about 6 weeks to a setpoint about 1000-fold lower at about 3–6 months. Once this setpoint is reached, a dynamic equilibrium is established between virus production and clearance. The half-life of individual SIV virions is <30 minutes in the absence of immunity and about 10 minutes in infected animals with an established immune response. It has been calculated that to maintain virus titers of 10^2 to 10^4 infectious virions per ml plasma requires the production of 10^{10} to 10^{12} new infectious virions daily. In this instance, high titer persistence is maintained by an extraordinary rate of virus production that exceeds the rate at which a potent cellular immune response can clear virus-infected cells.

Another example of apparent latency and activation is JC virus, a human polyoma virus that causes progressive multifocal leukoencephalopathy (PML), a slowly progressive but fatal illness. JC virus infects a high proportion of humans as determined by serosurveys. It is unclear whether JC virus is latent or causes a low-grade smoldering infection in humans, since it is hard to isolate from seropositive individuals and rarely causes PML. However, when humans are subject to long-term immunosuppression, the risk of PML rises dramatically, presumably as a result of the loss of protective cellular immunosurveillance. Recently, an increased incidence of PML has been seen in patients with AIDS and in patients with multiple sclerosis who are treated with a specific immunosuppressive monoclonal antibody.

Latency

Latent infections are produced by a considerable number of herpesviruses, including herpes simplex viruses (HSV), varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) of humans. There is a characteristic sequence of events following primary infection. Initially, the virus replicates in permissive cells at the portal of entry. The virus is lytic and destroys permissive target cells. Once immune induction has occurred, the virus is cleared and appears to be eliminated.

However, the viral genome persists in a latent form. Latency occurs in one or more cell types – such as neurons – that are distinct from the permissive cell types that support productive lytic infection. Neurons appear to be restrictive or permissive, depending upon their physiological state. Under conditions of restriction, the virus undergoes the early steps of entry and uncoating, but further steps in replication are blocked (see discussion of HSV below). In the absence of active replication, the viral genome is maintained in one of several forms, depending upon the specific virus and host cell. In some instances, the double-stranded DNA genome integrates into the host genome, while in other examples the genome persists as a non-integrated episome in the nucleus or cytoplasm.

If latency occurs in cell types such as neurons that do not divide, then there is no need to replicate the latent genome. If the latent genome is maintained in dividing cells with a finite life, then the genome must be replicated or it will be diluted to extinction. If the viral genome is integrated into the host genome, as with retroviruses, then it will be automatically replicated during the cell cycle. Episomal DNA can also be replicated by the enzymes

involved in copying cellular genomes. However, there are no parallel mechanisms for RNA, so RNA viruses cannot assume a latent state unless they undergo reverse transcription to DNA intermediates. Latent viral genomes can be detected by in situ polymerase chain reaction (PCR) methods that, at their most sensitive, can detect as little as one genome per infected cell (Figure 10.5).

Latency maintains the viral genome for the lifetime of the infected host. Activation of latent infections may occur at irregular intervals or it may never occur in some infected individuals. Activation of latent genomes can be initiated by a number of stimuli, characteristic for each virus. For instance, herpes simplex virus (HSV) can be activated by fever, sunburn and trigeminal nerve injury. Most of these stimuli appear to act upon the primary sensory neurons in which latent HSV genomes are maintained. However, on occasion, waning of the immune response can serve as a trigger for activation of some herpesviruses.

Following reactivation of HSV, the viral genome may be transported by axoplasmic spread in both centripetal and centrifugal directions. Centrifugal spread, toward the periphery, conducts the virus to the skin where it may replicate and spread, causing herpes labialis ('fever blister' or 'cold sore'). After spreading for a few days, host defenses prevent further spread and the skin lesion heals. Centripetal spread from the trigeminal ganglion conducts the HSV genome to the central nervous system, where, in some instances, it can spread to cause a devastating encephalitis.

Typically, viruses that cause latent infections induce a brisk and potent immune response that clears the initial infection. However, latently infected cells do not express viral proteins, permitting avoidance of immune surveillance. When the latent infection is activated, immune surveillance limits its spread. Latent viruses cannot be spread from host to host, but virus produced during activation may be spread to another host. For instance, activation of latent varicella zoster virus (VZV) produces characteristic skin lesions in older adults; seronegative children exposed to virus aerosolized from these lesions can develop chicken pox, the primary form of VZV infection.

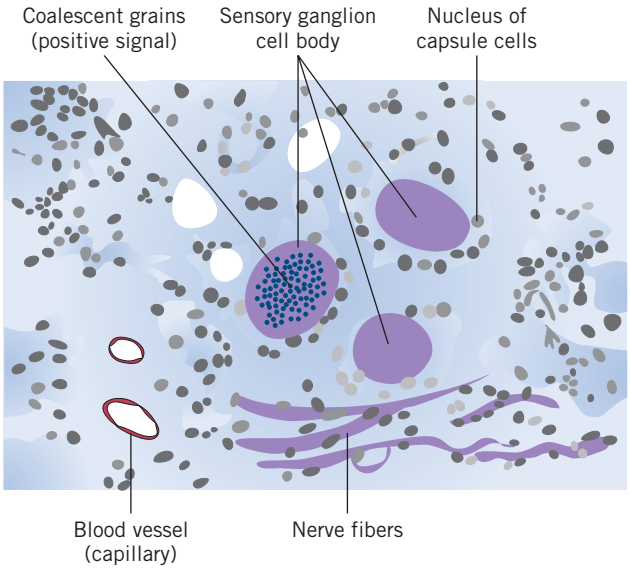

FIGURE 10.5 Demonstration of latent genomes. Herpes simplex virus (HSV) is maintained as a latent genome in first order sensory neurons whose cell bodies are located in sensory ganglia. This photomicrograph shows the large cell bodies of three sensory neurons and the grains mark the nucleus of one these cells that contains HSV gene sequences as detected by in situ hybridization. The probe detects RNA transcripts from the LAT (latency associated transcript) domain of HSV. After photographs supplied by Nigel Fraser, University of Pennsylvania.

Smoldering infections

'Smoldering' infections fall between the extremes of high titer persistence and latency. Infectious virus is produced, but at minimal levels that may require special methods for detection and isolation. Virus continues to spread from infected to uninfected cells but often at an indolent tempo. If the virus is pathogenic, it may produce a gradually progressive chronic disease. There is a detectable immune response to the virus and, in some instances, the response may be hyper-normal, due to the continuous presence of viral antigens. The ability of a virus to spread in the presence of a potentially effective immune response is a paradoxical phenomenon and involves a variety of strategies, several of which are described below.

Immunologically privileged sites

There are a few organs and tissues that appear to favor virus persistence, particularly the brain and kidney. The brain has classically been considered an immunologically 'privileged' site because immunological effector mechanisms may spare cells bearing foreign antigens if these cells are located in the brain (in contrast to foreign cells in other sites). This concept was originally enunciated by Medawar and collaborators who observed that grafts of allogeneic or xenogeneic tissues were more likely to survive in the brain than on the skin or at other sites. There are at least two factors that account for virus persistence in the brain. First, the blood–brain barrier limits the trafficking of lymphocytes through the brain and, second, neurons express little if any MHC class I molecules rendering them relatively poor targets for virus-specific cytolytic T lymphocytes (CTLs).

The kidney is the other major tissue that frequently harbors persistent viruses, such as JC and BK polyomaviruses and cytomegalovirus. Consistent with this observation, LCMV is cleared more slowly from the kidney than from other tissues, even the brain. However, there is no clear explanation why virus in the kidney should be able to evade immunological surveillance, although it has been speculated that lymphocytes may not readily cross the subendothelial basement membrane to access infected glomerular epithelial cells (see Figure 7.6 for anatomical relationships).

Intercellular bridges

In some instances, the process of entry of viruses into cells can be short circuited, so that a transient intercellular

bridge is formed permitting the viral genome to pass from cell to cell without having to survive in the extracellular environment (Figure 10.6), thus providing a means of avoiding neutralizing antibody. This phenomenon probably is operative in subacute sclerosing panencephalitis (SSPE), a progressive fatal disease. In SSPE, a defective variant of measles or rubella virus spreads gradually from neuron to neuron in spite of extraordinarily high titers of neutralizing antibody in the extracellular fluid of the brain parenchyma.

Suppression of MHC class I expression

A number of viruses, including adenoviruses and lentiviruses, encode specific proteins that are capable of downregulating the expression of MHC class I molecules (see Chapter 4). Virus-infected target cells are rendered relatively less sensitive to virus-specific CTL attack, permitting them to continue to produce virions for an extended period of time. Experimental deletion of the virus-encoded downregulating protein appears to reduce the ability of the virus to persist in vivo, suggesting that MHC class I damping may be a significant factor in persistence (see Figure 8.11).

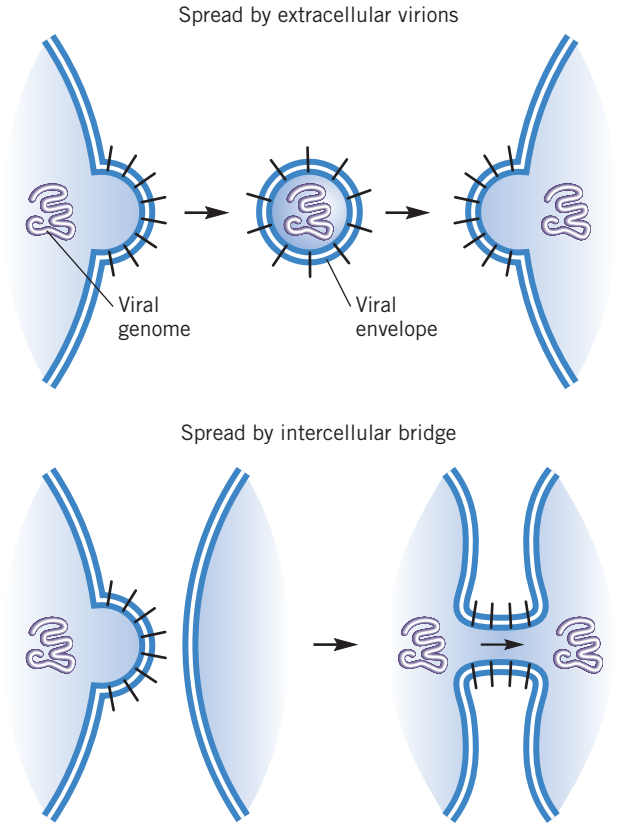

FIGURE 10.6 Cell-to-cell transmission of a virus by intercellular bridges, a hypothetical reconstruction. Top: normal cell-to-cell spread of an enveloped virus as free extracellular virions. Bottom: cell-to-cell spread of the viral genome through a transient intercellular bridge. This phenomenon has been invoked to explain the spread of measles and rubella viruses during persistent infection of the brain.

Infectious immune complexes

In some instances where a virus persists in the presence of an active immune response, infectivity in the blood circulates in the form of immune complexes that are composed of infectious virions coated by virus-specific antibodies. Immune complexes can be demonstrated by the addition of anti-IgG antisera that will 'neutralize' the infectivity (see Chapter 8). The molecular mechanism by which an antibody-coated virion can retain its infectivity has never been well elucidated. One possibility is that the complex is bound to Fc receptors on macrophages and internalized in vacuoles in which the complex dissociates, followed by infection of the macrophage. Consistent with this hypothesis, several of the persistent viruses (lymphocytic choriomeningitis virus, lactic dehydrogase virus and Aleutian disease virus), for which infectious complexes have been demonstrated, target macrophages as a major host cell.

Impaired cytolytic T lymphocyte (CTL) function

Recent advances have made it possible to quantify the function of CTLs according to their intracellular content of effector molecules such as IFNγ, TNFα and perforin (described in Chapter 6). If CTLs that recognize individual viral epitopes are sorted using tetramer staining, they vary in their content of effector molecules. It appears that HIV-specific CTL may be deficient in perforin content and this could provide an additional mechanism for escape from immune surveillance.

Antigenic variation

During the course of persistent infection, there may be a selection for viral variants that are able to escape neutralization. The ability of antibody to select for 'escape' mutants has been repeatedly documented in cell cultures. When a virus is plaqued in the presence of a single neutralizing monoclonal antibody, the titer is reduced drastically but characteristically some plaques occur at the frequency of $\sim 10^{-5}$. When these plaques are grown into virus stocks, they are resistant to neutralization (Figure 10.7) by the selecting monoclonal antibody. Such resistant virus variants usually represent point mutations, often in the viral attachment protein.

Neutralization escape mutants also play a role in some persistent infections of animals. This phenomenon has been observed with several persistent lentiviruses such as visna/maedi virus of sheep and equine infectious anemia virus. Equine infectious anemia virus (EIAV) is a lentivirus that produces a lifelong persistent infection of horses – the virus may be isolated from the blood even though the animals develop neutralizing antibody. Newly infected horses undergo discrete episodes of acute anemia, associated with bursts of viral replication. In each instance, the virus isolated during the episode of illness resists neutralization by serum obtained at the time of virus isolation (Table 10.4). However, the same serum can neutralize virus isolated at earlier times in infection. In other words, there is sequential replacement of virus

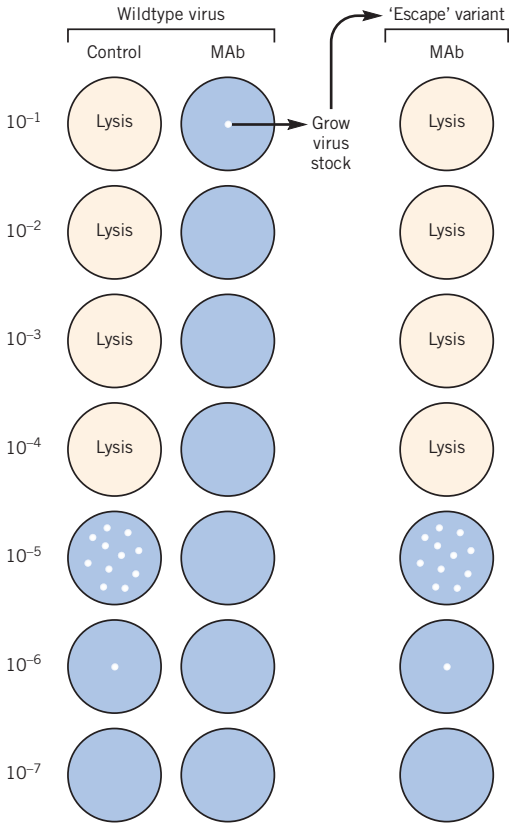

FIGURE 10.7 A monoclonal neutralizing antibody can select 'escape' mutants that are non-neutralizable. In this example, a virus is titrated in the absence and presence of a single monoclonal antibody that reduces the viral titer by 10^5. When the virus that escapes neutralization is grown into a stock, its titer is not reduced in the presence of the selecting antibody, indicating that it is an 'escape' mutant. Shaded area: cellular monolayer; 'hole', individual viral plaques; 'lysis', viral destruction of monolayer. After Gonzalez-Scarano F, Shope RE, Calisher CH, Nathanson N. Monoclonal antibodies against the G1 and nucleocapsid proteins of La Crosse and Tahyna viruses, in Calisher CH (ed.), *California serogroup viruses*, Alan R Liss, New York, 1983, with permission.

with newly emerging variants that can escape neutralization. Ponies that survive repeated episodes of illness finally develop such a broad neutralization response that they can suppress all potential variants, thereby modulating but not clearing the smoldering infection. A similar phenomenon occurs in HIV infections (discussed in Chapter 14).

The circumstances under which antigenic variants are selected in vivo have been studied using LCMV infection of mice. Ordinarily, when adult mice are infected with LCMV, the virus is cleared by CD8 cytolytic T lymphocytes (see Figure 10.4). However, CD8 'knockout' mice ($CD8^{-/-}$) also clear the virus, although less efficiently than intact animals. In these mice, clearance is mediated by neutralizing antibody, illustrating the synergistic role of the two main arms of the immune response. However, some weeks after clearance the virus may reappear (a phenomenon only seen in $CD8^{-/-}$ mice) and the re-emergence is then due to the outgrowth of escape variants that resist neutralization (Figure 10.8). When cloned, such variants can be shown to have a few mutations at sites that alter their neutralizing epitopes.

Escape mutants can also be selected by the cellular immune response. Most evidence for this phenomenon has been derived from somewhat contrived experimental models, but it is likely that similar mechanisms play a role in naturally occurring persistent infections. Figure 10.9 shows an example where variants of lymphocytic choriomeningitis virus (LCMV) were selected under pressure from specific antiviral CTL clones. The variants were sequenced and shown to bear mutations in the epitope against which the CTL clone was directed. In this example, there are three immunodominant CTL epitopes and a variant virus with mutations in two of these epitopes was compared with the wildtype virus for the ability to

Virus isolate (day of infection)	Fever spike (day of infection)	Neutralization index (log_{10}) of serum collected on the indicated day after infection					
		0 days	20 days	44 days	62 days	83 days	155 days
0 days		0	0	0.7	2.5	3.2	3.2
20 days	21	0	0	1.0	1.5	1.5	2.5
44 days	44	0	0	0	3.5	5.4	>5.4
62 days	62	0	0	0	0	2.0	2.0
83 days	83	0	0	0	0	0	3.5
155 days	155	0	0	0	0	0	0

TABLE 10.4 A smoldering infection associated with antigenic variation of the persistent virus. Equine infectious anemia virus (EIAV) infection is associated with periodic febrile episodes. The table shows data from one infected horse that experienced several fever spikes each lasting about 5 days. During each fever spike, virus isolated from the blood was tested for neutralizability. Each virus isolate was neutralized by sera collected after the time of the isolate but not by sera collected at or prior to the time of isolation. Likewise, each serum neutralized all the virus isolates made prior to the date of the serum but none of the isolates made thereafter. This is evidence of continual antigenic drift of the virus, which probably explains the burst of replication associated with each fever spike as well as the persistence of the virus in the face of an active neutralization response
Day 0: the virus used for infection; 0: negative. After Kono Y, Kobayashi K, Fukunaga Y. Antigenic drift of equine infectious anemia virus in chronically infected horses. *Archiv fur die gesamte Virusforschung* 1973, 41: 1–10, with permission.

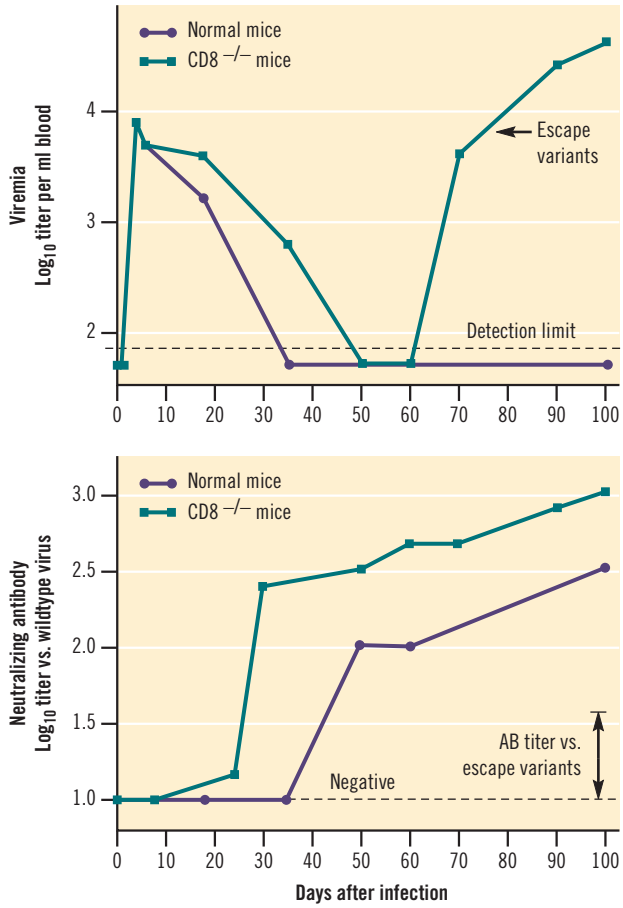

FIGURE 10.8 Selection of antibody escape mutants in vivo. In this model, lymphocytic choriomeningitis virus (LCMV) is used to infect normal and CD8 knockout ($CD8^{-/-}$) mice (the latter cannot produce effector CTLs). The normal mice clear virus rapidly, while the knockout mice clear virus but more slowly, mediated by neutralizing antibody. However, in some mice, the virus re-emerges weeks later. The re-emerging virus is an antibody escape variant that resists neutralization. After Ciurea A, Klenerman P, Hunziker L *et al.* Viral persistence in vivo through selection of neutralizing antibody-escape variants. *Proceedings of the National Academy of Sciences* 2000, 97: 2749–2754, with permission.

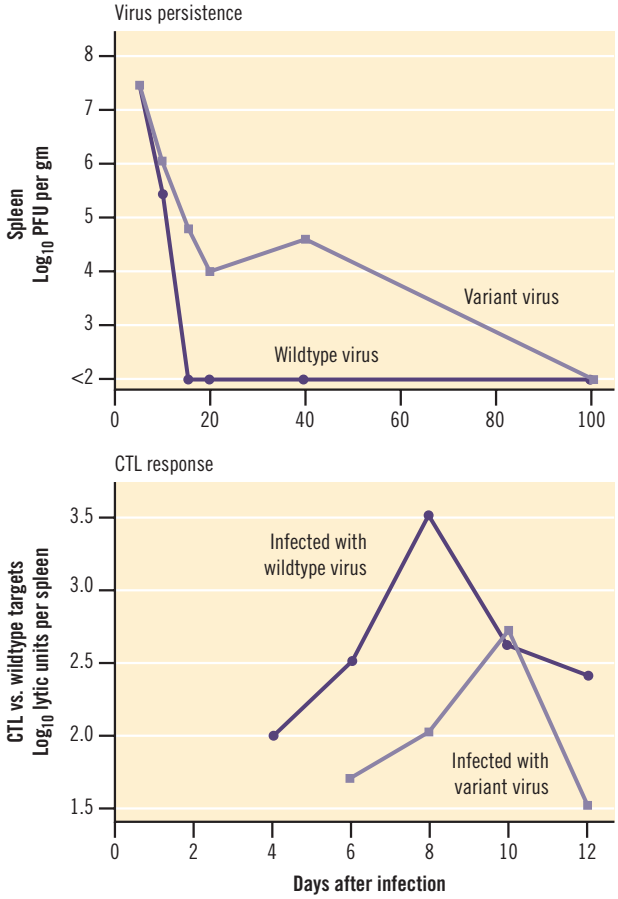

FIGURE 10.9 Persistent infection associated with viral variants that escape the cellular immune response. In this example, variant lymphocytic choriomeningitis viruses (LCMVs) were compared with wildtype parent virus for their ability to persist in adult mice after intravenous injection. Upper panel: the variant virus persists in the spleen for much longer than the wildtype virus. Lower panel: T lymphocytes from mice infected with the variant virus have much less CTL activity than do lymphocytes from mice infected with the wildtype virus when both are tested against target cells infected with wildtype virus. After Aebischer T, Moskophidis D, Rohrer UH *et al.* In vitro selection of lymphocytic choriomeningitis virus escape mutants by cytotoxic T lymphocytes. *Proceedings of the National Academy of Sciences* 1991, 88: 11047–11051, with permission.

persist after infection of adult mice. The double escape mutant (but not single mutants) persisted for much longer than wildtype virus, although it was eventually eliminated. In SIV infection of macaques – a model for HIV in humans – mutants that escape the cellular immune response have been well documented. In HIV infections, immune escape mutants play an important role in virus survival (see Chapter 14).

EXAMPLES OF PERSISTENCE

To provide a flavor of the diverse and subtle nature of persistent viruses, a few well-studied examples are described below. They illustrate different modes of persistence, including latency (herpes simplex virus), smoldering infection with demyelination (Theiler's virus), smoldering infection with progressive encephalitis (subacute sclerosing panencephalitis caused by measles virus) and high titer persistence with tolerance (hepatitis B virus).

Herpes simplex virus (HSV)

HSV represents a classic example of a virus that persists because of its ability to establish latent infections (Figure 10.10). HSV is an α-herpesvirus of humans that includes two serotypes: HSV-1 infects skin and mucous membranes of the mouth, while HSV-2 infects the genital mucosa. HSV is transmitted by skin-to-skin contact and initiates a productive lytic infection of epithelial cells. In the lytic cycle, three sets of viral genes are expressed in

sequential order: IE (immediate early), E (early) and L (late) genes.

During epithelial infection, the virus may enter the peripheral dendrites of first order sensory neurons, whose peripheral processes interdigitate among the epithelial cells. Fusion of the viral envelope with the plasma membrane of the neuronal process releases the viral nucleocapsid into the neuron, where it can be transmitted by retrograde axoplasmic flow to the cell body located in a sensory ganglion, such as the trigeminal ganglion. Sensory neurons are permissive for HSV and the virus may replicate in the ganglion with destruction of some cells. After 1–2 weeks, host immune defenses terminate productive infection in both skin and ganglion and the virus appears to have been cleared.

However, in some sensory neurons – but not epithelial cells – the virus establishes latency. The latent state is defined experimentally by the failure to recover infectious virus from a homogenized ganglion, while virus can be recovered from the same ganglion by co-cultivating explanted ganglion fragments with permissive cultured cells. Furthermore, in situ techniques can be used to demonstrate persistence of the viral genome, which is maintained as a closed circular double-stranded DNA non-integrated episome located in the nucleus of ganglion cells (see Figure 10.5). In a given ganglion, up to 5–10% of the sensory neurons may carry the latent genome and there may be up to 30 copies of the genome per infected cell. Latent HSV can also be detected in second order sensory neurons located within the central nervous system, where they could be one source of reactivation encephalitis.

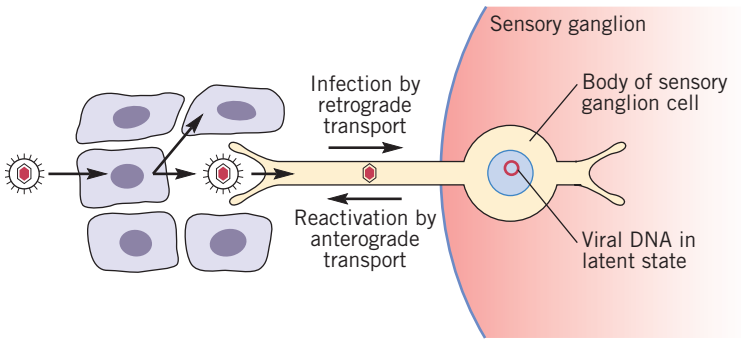

FIGURE 10.10 Life cycle of a persistent latent virus, herpes simplex virus (HSV). (1) HSV infects cells of the skin or mucous membranes where it causes a productive lytic infection. (2) Virions may enter the terminals of primary sensory neurons and the nucleocapsid of the virus is transported in a retrograde direction to the cell body – located in a sensory ganglion – where it can also undergo a productive lytic cycle. In some sensory neurons the virus becomes latent and the DNA genome is maintained in the nucleus as an episome. Latent HSV DNA undergoes limited transcription but no proteins are synthesized. (3) The latent genome may be activated, in which case a productive lytic infection is again initiated and nucleocapsids can be passively transported in an anterograde direction, delivering the virus to the epithelium where it can once again replicate to produce a 'cold sore' or 'fever blister'. HSV is shown as a nucleocapsid surrounded by an outer envelope. After Ahmed R, Morrison LA, Knipe DM. Viral persistence, in Nathanson N, Ahmed R, Gonzalez-Scarano F *et al.* (eds), *Viral pathogenesis*, Lippincott Raven Publishers, Philadelphia, 1997, with permission.

The molecular mechanisms that determine whether HSV will become latent or complete its full replicative cycle are not well understood, but the metabolic state of the infected sensory neuron is undoubtedly a major determinant. Either the neuron fails to provide sufficient factors to permit expression of viral genes or produces proteins that inhibit the expression of these genes. No individual viral genes appear to be essential for establishment of latency, since a wide variety of HSV mutants, each lacking specific viral genes, can establish latency and subsequently be recovered by growth of ganglion explants under conditions permissive for the mutant virus. For instance, ts mutants of HSV, that are restricted at the body temperature of the mouse (37°C), can establish latency after peripheral infection and be recovered from sensory ganglia explanted at the permissive temperature (33°C).

During latency most viral genes are not transcribed, with the exception of the LAT (latency associated transcripts) genes, one small segment of the HSV genome. However, the LAT transcripts, which encode two open reading frames, are never translated. Furthermore, LAT-negative mutants can establish latency, although less efficiently than wildtype virus. The function of the LAT genes and their relevance to latency remains enigmatic, but it appears that they have at least two functions, to protect the infected neuron from virus-induced apoptosis and to suppress the action of immediate early genes that initiate lytic infection.

Latent HSV persists life long and may be periodically reactivated in some infected hosts while others never experience reactivation. During reactivation, the virus initially replicates in the sensory ganglion. Viral nucleocapsids are then passively transported in the anterograde direction to the skin where they can produce a lytic infection, similar in character to the initial infection. Again, this infection is brought to a close by host immune defenses. Rarely, following reactivation, HSV initiates an acute and – prior to the development of antiviral drugs – often fatal encephalitis. It is not known whether reactivation encephalitis arises from virus transported from the sensory ganglia (first order sensory neurons) or from virus latent in second order sensory neurons within the central nervous system.

A variety of natural or experimental insults can lead to reactivation, including fever, sunburn, stress, hormonal changes, immunosuppression or trauma to the trigeminal nerve. It is postulated that reactivation of latent HSV is initiated by a physiological change in the sensory neurons harboring the latent genome. Under this view, reactivating stimuli lead to upregulation of cellular proteins, such as transcriptases, that directly or indirectly initiate transcription of the HSV IE genes. Alternatively, activation might involve release of a hypothetical inhibitor of HSV replication. The specific cellular factors involved in activation have yet to be defined. In addition, certain viral genes appear to modulate reactivation, particularly the LAT genes and a protein designated ICP0 (infected

cell protein 0), since viral mutants lacking these genes are more difficult to reactivate in vivo.

It should be noted that there is an alternative hypothesis that suggests that during 'latency' there is a continual low level of replication limited to a few ganglion cells. Under this view, virus-specific T cells in the infected ganglia control the level of replication and activation, in some instances, is triggered by a release of immune surveillance. There are several lines of evidence for this view, including the presence of epitope-specific CD8+ T cells at the sites of latency and the failure to establish latency in mice with various types of experimental immunodeficiency. It seems likely that persistence involves both immune control of lytic virus replication and a molecular mechanism that maintains the viral genome in a state of latency.

Picornaviruses (Theiler's virus)

Theiler's murine encephalomyelitis virus (TMEV) is an example of a virus that persists as a smoldering infection in the presence of an apparently brisk and intact immune response. TMEV is an enzootic picornavirus (small RNA virus) of wild mice that spreads in nature as an enteric virus, being excreted in the feces and contracted by ingestion. Wildtype isolates of TMEV fall into two distinct groups, virulent and persistent, based on their biological properties (Table 10.5). After intracerebral injection, the virulent strains replicate to high titer in neurons of the brain and spinal cord and produce a rapidly progressive fatal encephalomyelitis. Intracerebral injection of sublethal doses of virulent strains cause a transient CNS infection but the virus is always cleared by immune defenses and never persists. The persistent strains of TMEV cause an acute but sublethal encephalitis due to an infection of neurons, similar in character but less widespread than that caused by the virulent strains. In contrast to the virulent strains, the persistent strains of TMEV are never cleared from the CNS and low titers of infectious virus can be isolated without requiring special methods such as co-cultivation.

During the persistent phase of CNS infection, there is a dramatic change in the localization of TMEV. The virus disappears from the gray matter (part of the CNS where most neurons are located) and moves into the white matter (part of the CNS where nerve fibers are concentrated) and infects glial cells (supporting cells of the CNS) and microglia (macrophages of the CNS). During the transition from acute to persistent infection (about 15 days after infection) viral titers decrease in the brain, but viral RNA levels increase in the spinal cord. During persistence, very active replication continues, sufficient to generate $>10^9$ viral RNA copies per day, although infectious titers are modest. The infection of oligodendroglia destroys some of the myelinated sheaths that surround nerve fibers and this demyelination causes neural dysfunction, manifested by a waddling gait and incontinence.

In spite of the marked biological differences between virulent and persistent strains of TMEV, their RNA sequences are 90% identical and their amino acid sequences are 95% identical, illustrating the subtle differences that can exist between persistent and non-persistent viruses. To elucidate the genetic determinants of virulence and persistence, chimeric viruses have been constructed that combine different domains of the genomes of the two groups of viruses. Full expression of virulence appears to require the capsid structural proteins and the 5' non-coding domain of the genome. Substitution of individual segments (any of four segments that spanned the whole genome) of the virulent viral genome into the genome of the persistent virus produced chimeric viruses, all of which had markedly reduced acute virulence, persisted and caused demyelination. This suggests that the virulent TMEV strains are potentially capable of persisting and causing demyelination, if mice survived the acute phase of infection.

The immune response plays a dual role in TMEV infection. TMEV elicits brisk neutralizing antibody and CTL responses. Clearance of the virus after the acute phase is mediated by the synergistic effect of antibody, CD4+ and CD8+ T cells and abrogation of any of these modalities impairs the control of CNS virus. On the other hand, demyelination is associated with an infiltration into the white matter of B and T cells (both CD4+ and CD8+ lymphocytes) and depletion of immune cells reduces lesion severity. There is no evidence that persistence is due to the failure of immune surveillance, since persistent strains of TMEV induce an active immune response.

The mechanism(s) of persistence of TMEV has been difficult to elucidate. During persistence, TMEV is confined to glia and macrophages, both of which are somewhat restrictive and produce low levels of viral capsid proteins and little free infectious virus. This may make

Property	Virulent (GDVII) subgroup	Persistent (TO) subgroup
Relative lethality after intracerebral injection	10 000	1
Acute polioencephalitis	Marked, lethal	Moderate, sublethal
Demyelination	No	Yes
Persistence in the CNS	No	Yes
Plaque size	Large	Small
Temperature sensitive in cell culture	No	Yes

TABLE 10.5 A comparison of virulent and persistent variants of Theiler's murine encephalomyelitis virus (TMEV). The two groups are named after prototype isolates, the virulent GDVII and the less virulent persistent TO isolate and there are a number of wildtype isolates of each group
After Tsunoda I, Fujinami RS. Theiler's murine encephalomyelitis virus, in Ahmed R, Chen I (eds), *Persistent viral infections*, Wiley, New York, 1999, with permission.

infected cells less susceptible targets of the humoral and cellular immune response. Persistence does not appear to be associated with selection of viral variants, such as antigenically altered escape mutants, although a few neutralization escape mutants have been observed. TMEV presents the enigma of a virus that continues to undergo active replication and spread in the presence of a brisk immune response that, in theory, should be able to clear the virus.

Morbilliviruses (measles)

Subacute sclerosing panencephalitis (SSPE) is an example of a smoldering infection – due to measles virus – that persists in the face of a supernormal immune response. In this example, escape from immune surveillance is associated with the selection of variant viruses that lose the ability to mature and bud properly while maintaining the gene functions for replication of the viral nucleocapsid. Measles virus is a human morbillivirus consisting of an envelope enclosing a nucleocapsid containing a single-stranded RNA genome of negative polarity. The genome encodes six proteins: the nucleocapsid (N), phosphoprotein (P) and large – polymerase – (L) protein are associated with the viral RNA, while the hemagglutinin (H) and fusion (F) proteins are inserted into the viral envelope and the matrix (M) protein binds the viral core to the envelope during virion maturation, which is accomplished by budding through the plasma membrane. SSPE variant viruses exhibit under-expression or defects in one or more of the M, F and H proteins, inhibiting budding and the production of free infectious virions (Figure 10.11).

Measles is an ubiquitous virus that spreads to children by the respiratory route and causes a systemic febrile infection with a rash that usually resolves in 1–2 weeks with no serious consequences. However, one rare (~1 case per 100 000 primary infections) complication of measles is SSPE, which occurs unpredictably in apparently normal children following uneventful recovery from acute measles. Several years after measles, these children develop a progressive subacute encephalitis that is invariably fatal in 6–12 months. On brain biopsy or at autopsy, measles antigens can be detected in the brain and electron microscopy reveals measles nucleocapsids in neurons and glial cells in the brain.

It is very difficult to isolate measles virus from the brain tissue of patients with SSPE, except by special methods involving explantation and co-cultivation. Furthermore, viral isolates are usually defective, with most defects localized to the matrix protein or the viral envelope proteins, so that SSPE isolates can be considered to be 'maturation defective' virus variants. As such, they probably spread in vivo by intercellular bridges (see Figure 10.6). During the disease, the patients exhibit supernormal virus-specific immune responses, with very high levels of measles antibodies in their blood and spinal fluid. Although these antibodies are able to neutralize normal measles virus, they cannot interrupt the spread of SSPE variant viruses, which fail to form free infectious virions. In summary, it appears that SSPE viruses are defective variants that spread very poorly but can elude neutralizing antibody. As a result they cause a slowly progressive encephalitis that is eventually fatal.

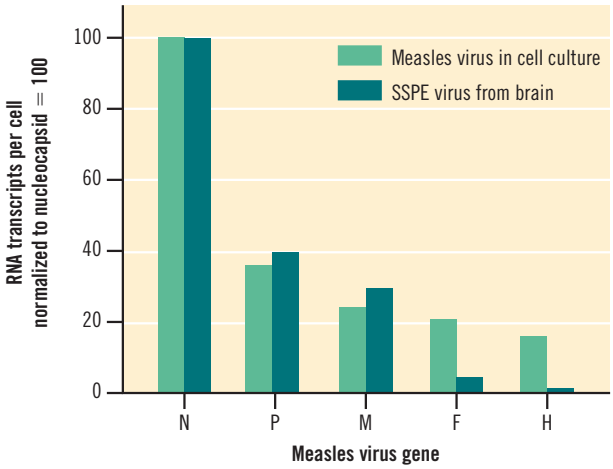

FIGURE 10.11 Persistent measles virus infection of the brain (SSPE, subacute sclerosing panencephalitis) is associated with reduced and defective virus replication. The figure compares the per cell expression of five viral RNAs plotted according to their order of transcription (3' to 5' in this negative strand virus). Viral RNA in a lytic cell culture is compared with that in the brain of a patient with SSPE. In SSPE there is marked reduction in all viral mRNAs (not shown in this representation) and the 'gradient' of RNA transcription is much steeper, resulting in a relative paucity of the 'downstream' RNAs which encode the viral envelope (F and H proteins). This pattern explains the defectiveness of the SSPE virus genome which produces few if any infectious virions. N: nucleocapsid; P: polymerase; M: matrix; F: fusion; H: hemagglutinin. After Cattaneo R, Rebman G, Baczko K, ter Meulen V, Billeter MA. Altered ratios of measles virus transcripts in diseased human brains. *Virology* 1987, 160: 523–526, with permission.

Hepatitis B virus (HBV)

HBV infection follows different courses, depending upon the age of infection. Infection of adults is often a self-limited acute process, with clearance of the virus often accompanied by hepatitis. Neonatal infection (transmission from a woman who is a chronic virus carrier to her newborn infant) usually leads to persistence and the following description pertains to that situation (Figure 10.12). The infected infant has high levels of circulating HBsAg and serum HBV DNA. There is no anti-HBs or anti-HBe antibody (to viral surface or precore antigens), but anti-HBc antibody (to viral core antigen) is induced, a state sometimes called 'split tolerance'.

Persistence is associated with high dose cellular tolerance against HBsAg, with only minimal levels of HBs-specific CD8+ T cells due to the deletion or exhaustion of antigen-specific T lymphocytes. Since CD8+ T cells have been shown to play a major role in control or clearance of HBV, this is a major factor in persistence. In addition, HBeAg (or precore protein), one of the proteins encoded by HBV, is thought to have an immunosuppressive effect,

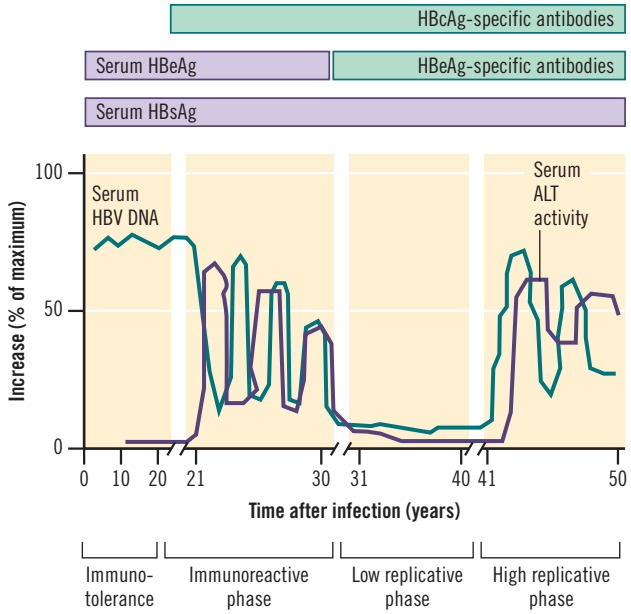

FIGURE 10.12 The course of persistent hepatitis B virus following infection of a newborn infant. Most HBV infections begin as an immunotolerant process with high virus titers and a minimal cellular response which may last for decades to a lifetime. However, for reasons that are not known, the infection may shift into one of three other modes, an immunoactive phase, a low replicative phase or a high replicative phase. In the low replicative phase, patients may control or clear the infection with the development of anti-HBe antibody and recovery of cellular immunity. In the immunoactive or high replicative phases, the immune response attempts to clear the virus, but is only partially successful, with resultant necroinflammatory hepatitis that may progress to cirrhosis (permanent liver damage). ALT: alanine transaminase, a liver enzyme whose level in the serum reflects liver function. After Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nature Reviews Immunology* 2005, 5: 215–229, with permission.

contributing to the establishment of tolerance and persistence.

High titer persistence may last for decades to a lifetime. However, for reasons that are not known, the infection may shift into one of three other modes, an immunoactive phase, a low replicative phase or a high replicative phase. In the low replicative phase, patients may control or clear the infection with the development of anti-HBe antibody and recovery of cellular immunity. In the immunoactive or high replicative phases, the immune response attempts to clear the virus but is only partially successful, with resultant necroinflammatory hepatitis that may progress to cirrhosis (permanent liver damage).

DISEASES ASSOCIATED WITH PERSISTENT INFECTION

Persistent virus infections can be asymptomatic, as in the case of hepatitis B and many of the latent herpesviruses. However, persistent infections are often associated with chronic progressive illnesses that are quite diverse, although individual viruses usually produce a stereotyped and limited spectrum of disease. Table 10.6 lists some examples of the different kinds of diseases associated with virus persistence. High titer persistence is often associated with circulating immune complexes, which eventually produce glomerulonephritis or vasculitis due to complex deposition in and around the walls of blood vessels. Latent herpesviruses are periodically activated, replicate and cause lytic infections in permissive cells. The nature of the consequent disease depends upon the permissive cell type and includes destructive lesions of the mucosa or skin (HSV, VZV), pneumonitis (CMV), retinitis (CMV), encephalitis (HSV) and mononucleosis (EBV). Smoldering infections of the brain cause progressive chronic destructive encephalitis or myelitis (measles virus, CDV, JC polyoma virus, HTLV I) that may be accompanied by demyelination (TMEV, VMV, CDV, HTLV I). Other smoldering infections are associated with diverse illnesses, such as AIDS (HIV, SIV, FIV) or episodic acute anemia (EIAV). A number of persistent viruses are oncogenic and cause a wide variety of neoplasms (discussed in Chapters 11 and 12).

REPRISE

Most viruses cause acute self-limited infections that are cleared by the immune response. However, a number of viruses from a wide variety of virus families, can persist for months, years or life long. In order to persist, a delicate balance must be achieved so that the host is not killed by the destructive effects of the virus and the virus manages to evade immune surveillance. Most persistent infections fall into one of three distinct patterns:

1. Latent infections, in which the virus persists as an untranslated genome that eludes recognition by the host immune response. Latent infections may reactivate periodically, with viral replication and disease manifestations and reactivations are usually terminated by host immune surveillance.
2. Persistence of the virus at high titer in various tissues, usually associated with immune tolerance due to deletion or exhaustion of antigen-specific T lymphocytes. Most high titer persistent infections are caused by non-lytic viruses, so that infected cells can continue to shed virus over long periods but, in a few instances, lytic viruses persist at high titer in tissues whose cells regenerate at a rate sufficiently rapid to compensate for cell killing.
3. Smoldering infections fall between the other two patterns, with the virus continuing to replicate but at a low level in the face of a brisk immune response. Evasion of immune surveillance is achieved by a number of mechanisms including antigenic variation, infectious immune complexes, immunologically privileged sites, intercellular bridges and downregulation of MHC class I expression.

Persistent infections may be asymptomatic or may be associated with a wide variety of chronic diseases.

Virus family	Virus	Host	Disease
High titer persistence			
Arena Parvo	LCMV Aleutian disease	Mice Mink	Glomerulonephritis, vasculitis Glomerulonephritis, vasculitis
Latent infections			
Herpes	HSV CMV EBV VZV	Humans Humans Humans Humans	Cold sores, encephalitis Pneumonitis, retinitis, encephalitis Mononucleosis Herpes zoster
Smoldering infections			
Morbilli	Measles CDV	Humans Dogs	Subacute sclerosing panencephalitis Encephalitis, demyelination
Retro	HTLV I	Humans	Tropical spastic paraparesis Adult T cell leukemia
Polyoma	JC	Humans	Progressive multifocal leukoencephalopathy
Lenti	VMV EIAV HIV	Sheep Horses Humans	Interstitial pneumonitis, demyelination Episodic hemolytic anemia AIDS
Oncogenic viruses			
Retro	MuLV	Mice	Hematopoietic, lymphoreticular neoplasms
Hepadna	HBV	Humans	Hepatocellular carcinoma
Papilloma	HPV	Humans	Cervival carcinoma
Herpes	EBV	Humans	Burkitt's lymphoma

TABLE 10.6 Selected examples of diseases associated with persistent viral infections
CDV: canine distemper virus; CMV: cytomegalovirus; EBV: Epstein-Barr virus; EIAV: equine infectious anemia virus; HBV: hepatitis B virus; HSV: herpes simplex virus; HTLVI: human T cell leukemia virus type I; JCV: JC virus; LCMV: lymphocytic choriomeningitis virus; HPV: human papilloma virus; MuLV: murine leukemia virus; VMV: visna maedi virus; VZV: varicella zoster virus.

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Chapter 11

Viral Oncogenesis: Retroviruses

Neal Nathanson and Harriet L. Robinson

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ONCOGENIC VIRUSES OF HUMANS AND ANIMALS

A large number of viruses, from several families, have oncogenic potential in animals and some of these have representatives among the viruses that infect humans. Most oncogenic RNA viruses belong to the family Retroviridae (Table 11.1) and the following account will focus on a few of the oncogenic retroviruses that illustrate the mechanisms of transformation and tumorigenesis. Much more information can be found in the books and reviews listed at the end of this chapter.

Cellular immortalization and transformation

Neoplasia is a descriptive term that denotes an abnormal tissue overgrowth that may be either localized or disseminated. When cells from neoplastic tissue are grown in culture, they exhibit several characteristics that differentiate them from normal cells, particularly immortalization and transformation. Normal cells, such as fibroblasts, can be maintained in culture for about 50 divisions, after which they undergo 'senescence', lose their capacity

Host	Virus and disease	Oncogenic mechanism(s)
Human	HTLV-I Adult T cell leukemia	Tax protein drives cell cycle, activates NFκB, blocks apoptosis
Animal	BLV Bovine leukemia	Tax protein drives cell cycle, activates NFκB, blocks apoptosis
	MuLV Murine leukemia	Promoter insertion, enhancer activation
	RSV Avian sarcoma	Viral oncogene (*src*)

TABLE 11.1 Representative oncogenic retrovirus diseases of animals and humans
BLV: bovine leukemia virus; HTLV-I: human T cell leukemia virus type I; MuLV: murine leukemia virus; RSV: Rous sarcoma virus.

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for division and die out. Immortalization is the ability to undergo an unlimited number of cell divisions and be maintained in culture indefinitely. Immortalized cells must undergo further changes before they exhibit the transformed phenotype. Transformation often includes the ability to overgrow in cell culture, in contrast to untransformed cells which show contact inhibition and grow as monolayers. Transformed cells may form colonies in soft agar, while normal or immortalized cells cannot, and may produce tumors when transplanted into normal or immunodeficient animals.

STRUCTURE AND REPLICATION OF RETROVIRUSES

Retroviruses are enveloped viruses with a core containing a single-stranded RNA genome of positive polarity. The envelope is composed of a lipid bilayer into which is inserted a single glycopeptide that is post-translationally cleaved into a transmembrane (TM) protein non-covalently linked to a surface (SU) protein, and these heterodimers are usually arranged in a trimer. The core consists of two copies of the viral genome associated with a nucleocapsid (NC) protein, enclosed in a capsid composed of a capsid (CA) protein, a matrix (MA) protein and a phosphorylated protein (p12).

A prototypical simple retrovirus has three principal genetic domains. The *gag* (group-specific antigen) gene encodes four proteins that compose the core of the virus. The *pol* (polymerase) gene encodes three enzymes, the protease that cleaves the gag polyprotein into its constituent four peptides, the reverse transcriptase that transcribes the RNA genome into its DNA complement and the integrase that catalyses the integration of the double-stranded DNA copy into the host genome. The *env* gene encodes the two envelope proteins. In addition, in the RNA there are specific cis-acting sites for critical steps in replication, such as a primer binding site to initiate reverse transcription, a packaging site for incorporation of the genome into virions and a site for 3' polyadenylation of nascent mRNA molecules. The genomic organization of a simple retrovirus is shown in Figure 11.1.

Retroviruses are distinguished by their mode of replication, which employs reverse transcription to synthesize a DNA intermediate (the provirus) that integrates into the host genome prior to further steps in viral replication. Unique sequences at the 5' and 3' ends of viral RNA contribute to long terminal repeat (LTR) sequences at each end of the provirus that provide the transcriptional control elements for provirus expression. Transcription of the provirus produces both RNA messages and new RNA genomes (Figure 11.1). This mode of replication has several important consequences. Integration of the DNA intermediate into the genome of the host cell offers the possibility of perpetuating the viral genome through the lifetime of the host and even, under some circumstances, in the germ line of the host. Reverse transcription is not monitored by the cellular enzymes that edit DNA, permitting high rates of mutations.

The integration of viral DNA also provides a possibility to disrupt host genes or to alter their expression by up- or downregulation via the insertion of promoters, enhancers or transcription termination sequences that are contained within the retroviral LTRs. In addition, integration into cellular DNA facilitates recombination with cellular sequences that can be added to or substituted for segments of the viral genome. Some cellular genes acquired by recombination can confer transforming ability upon the virus. If recombinant retroviruses lose segments of their own genome they may become defective, depending for their replication upon non-defective replication-competent 'helper' retroviruses that must co-infect the host cell to permit the defective virus to complete its life cycle.

The other essential feature of retroviruses is that, in general, they are not cytocidal. They mature by budding through the plasma membrane and this need not compromise the vital functions of the host cell. Also, none of the retroviral proteins appear to induce apoptosis. The innocuous nature of viral replication and the integration of the DNA provirus lead to life-long associations of virus and cell, which promote certain modes of cellular transformation. In some instances, infection of the host is from the mother, either via the germline or soon after birth, so that the infected host is likely to be tolerant of the neo-antigens encoded by the virus, further enhancing the possibility of high titer persistent infection.

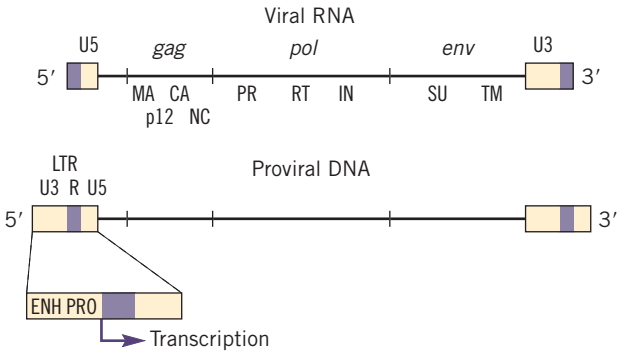

FIGURE 11.1 Organization of the genome of a typical simple retrovirus. The RNA genome is about 9 kb long and is bounded at both ends by a non-coding repeat (R) region that encloses the three major coding genes, the *gag* (group antigen), *pol* (polymerase) and *env* (envelope) genes. The diagram indicates the position of the major proteins encoded by each of these genes, including the MA (matrix), p12, CA (capsid), NC (nucleocapsid) proteins of gag, the PR (protease), RT (reverse transcriptase), IN (integrase) enzymes of pol and the SU (surface) and TM (transmembrane) proteins of env. During replication, the RNA genome is copied by the viral reverse transcriptase into a DNA complement of the genome (provirus). In the process of reverse transcription, the DNA copy acquires a complete LTR (long terminal repeat) sequence at both its ends. The LTR is divided into three domains, the U3 (unique 3′), R (repeat) and U5 (unique 5′) regions. For virus replication, the DNA genome is transcribed back into RNA. Transcription begins at the upstream end of the R domain and proceeds through the genome, terminating in a polyadenylation signal at the downstream end of the 3′ LTR. The U3 domain contains basal promoters (PRO, such as a TATA box) and upstream enhancers (ENH) that drive transcription. The enhancers are regulatory elements in DNA that bind cellular proteins that act to 'open' condensed DNA (DNA associated with histones) so that it is accessible to the polymerase complex.

Category	Occurrence (incubation period)	Mechanism of transformation (clonality)	Replication competence	Examples
Non-acute transforming	In nature, more common (years)	Insertional upregulation of cellular proto-oncogenes (clonal)	Competent	MuLV ALV FeLV
Acute transforming	In nature, uncommon (weeks)	Action of viral oncogenes (polyclonal)	Defective Requires helper virus	ASV MSV FeSV
Trans-acting transforming	In nature, uncommon (years)	Transactivation by viral accessory genes (oligoclonal)	Competent	HTLV 1 BLV

Table 11.2 Major categories of oncogenic retroviruses. Viral oncogenes are derived from cellular proto-oncogenes, but are usually modified by the structural alteration or loss of domains involved in regulation of proto-oncogene expression
Abbreviations: ALV: avian leukemia virus; ASV: avian sarcoma virus; BLV: bovine leukemia virus; FeLV: feline leukemia virus; FeSV: feline sarcoma virus; HTLV: human T cell leukemia virus; MuLV: murine leukemia virus; MSV: murine sarcoma virus.

MECHANISMS OF ONCOGENESIS

There are three major classes of oncogenic retroviruses (Table 11.2), the non-acute transforming viruses, the acute transforming viruses and the trans-acting viruses. There are distinct differences in the genome structure of each group, differences that play a central role in their oncogenic activity and other biological properties (Figure 11.2). This introductory treatment describes only a few examples of these patterns of oncogenesis (see Further Reading).

Certain common themes are present in all of the transforming retroviruses. Tumor formation is generally a multistep process that involves steps initiated by the virus and other steps that are not virus-mediated. Often, tumor induction requires activation of more than one cellular oncogene. In addition to the over-expression of oncogenes, downregulation of cellular tumor suppressor genes frequently plays a role in the multistep pathway to tumor induction. Each oncogenic virus tends to produce a characteristic narrow range of tumors, related to the cells that it infects and the cell-specific activity of the enhancers in its LTR or its transduced oncogene.

Non-acute transforming retroviruses: insertional mutagenesis

The non-acute transforming retroviruses are found in a number of species, particularly mice, chickens and cats, but there are no human representatives of this virus group. The following description is based on the murine leukemia viruses (MuLV), the examples that have been studied in the greatest detail. MuLV are replication competent simple retroviruses that are limited in their host range to cells that express their specific receptor. The DNA provirus integrates at many sites in the genome of the infected cell and persists for the life of the infected cell.

Transformation is due to the effect of the LTR of the provirus upon expression of host genes. The effect can be exerted in two distinct ways (Figure 11.3), although there are several variations in these routes to transformation.

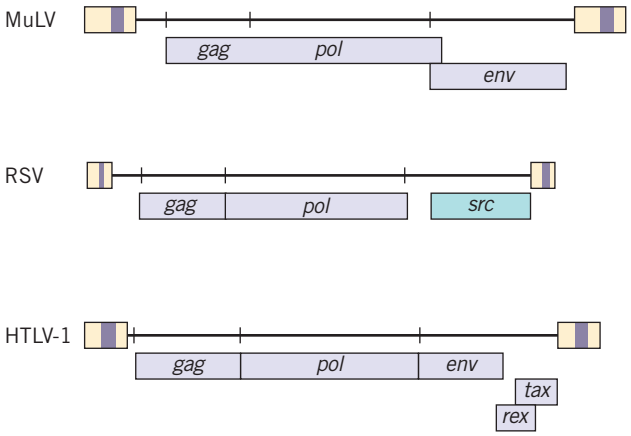

FIGURE 11.2 Organization of the genomes of different groups of oncogenic retroviruses. Three different groups of viruses are distinguished in this figure, shown in the form of DNA proviruses. Open rectangles indicate the messages for the principal genes, offset vertically if they are overlapping. Top: the non-acute transforming viruses have the organization of simple retroviruses (see Figure 11.1), are replication competent and transform by promoter or enhancer insertion. Middle: The acute transforming retroviruses usually carry an additional gene (oncogene) that they have acquired by recombination with cellular proto-oncogene sequences. As a result, these viruses lose viral genes and require a 'helper' virus that encodes the missing viral genes to form infectious virions. Bottom: the trans-acting retroviruses have the complete genomic content of a simple retrovirus and are replication competent, but they also contain accessory genes, the most important of which are the *rex* and *tax* genes; both *rex* and *tax* are required for replication and *tax* also confers oncogenic properties upon these viruses. MuLV: murine leukemia virus; RSV: Rous sarcoma virus; HTLV-I: human T lymphocyte tropic virus type I.
After Coffin JM, Hughes SH, Varmus HE (eds). *Retroviruses*, Cold Spring Harbor Press, 1997, with permission.

If the provirus is integrated upstream from a cellular gene and transcribes toward the cellular gene, then the proviral promoter can initiate transcription that reads through into the cellular gene, increasing the transcription of the cellular gene (*promoter insertion*). If the cellular gene that is upregulated has an influence upon cellular growth, then transformation may result. The resulting tumors are usually clonal but the transformed target cell will depend upon the genomic site of insertion.

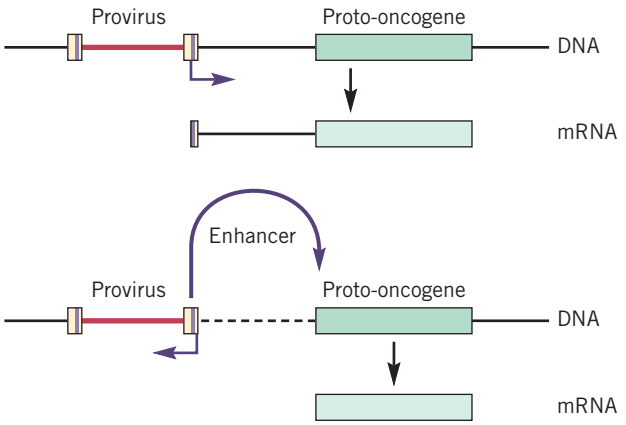

FIGURE 11.3 Mechanisms of transformation by non-acute transforming retroviruses. Top: insertional activation. The DNA provirus is integrated upstream and in the same orientation to a proto-oncogene, a cellular gene whose product can alter cellular growth by one of many possible mechanisms. Transcription is initiated in an LTR of the provirus and reads through the downstream cellular gene, increasing its rate of transcription. Bottom: enhancer activation. The DNA provirus is inserted either 3′ or 5′ (insertion can be >100 000 bp away) of a cellular gene that influences growth. For 5′ insertion, provirus transcription is in the upstream direction away from the cellular gene. For 3′ insertion, provirus transcription is in the same orientation as the proto-oncogene and downstream from it. The enhancers are regulatory elements in DNA that bind modulatory factors, some of which act to 'open' condensed DNA (DNA associated with histones) so that it is accessible to the polymerase complex. The bent arrows indicate the direction of transcription from the viral LTR. After Fan H. Murine leukemia virus, in Ahmed R, Chen I (eds), *Persistent viral infections*, Wiley, New York, 1999, with permission.

Alternatively, if the provirus is located near a cellular gene, but is oriented to read away from the cellular gene, the enhancer sequences in the provirus may bind cellular factors that 'open' condensed DNA and enhance transcription of neighboring cellular genes regardless of their orientation (*enhancer activation*). Again, if the gene influences cellular growth, transformation may result. Enhancer activation is a more frequent phenomenon than promoter insertion, probably because it can occur at more sites in the cellular genome.

Detailed studies have been made of many leukemias and other cancers in mice infected with various non-acute transforming retroviruses. Based on these studies, catalogs have been constructed of the cellular genes that are upregulated. This work has identified many genes that influence cell growth and division, each of which can produce a transformed phenotype.

The likelihood that an MuLV will integrate in the cellular genome in a position leading to transformation is very low and many integration events occur for each instance of transformation. The type of tumors that develop reflect the function of the upregulated gene and a particular virus can cause different tumors depending on the chance site of insertion. The potential for tumor induction is directly correlated with the number of integration events and those MuLV strains that replicate to highest titer in mice tend to be the most leukemogenic. Thus, infection of newborn (compared to adult) animals carries a higher risk of leukemia, partly because MuLV replicates to higher titers in young animals and partly due to immunological tolerance.

The other critical point is that cellular transformation by a non-acute retrovirus is not sufficient to cause leukemia. Rather, there must be additional subsequent oncogenic events that are described later in this chapter. The combined low probability of insertional mutagenesis of proto-oncogenes and the requirement for subsequent genetic events explain the clonal nature of the resulting tumors.

In summary, the salient features of the non-acute transforming retroviruses (see Table 11.1) are the replication competence of the virus, transformation via insertional mutagenesis and upregulation of a proto-oncogene, the ability to cause different types of tumors by inserting into different proto-oncogenes, the long incubation period and the clonal nature of the transformed cells.

Under special circumstances, retroviruses can also cause oncogenic insertional mutagenesis in humans. Retrovirus vectors have been successfully used to insert genes into human myeloid cells, as gene therapy to correct specific genetic defects. Since these vectors can insert randomly into the target genome, there is the potential for upregulation of a proto-oncogene, which could lead to neoplasia. This sequence of events has been recently reported in children who were treated for a genetic defect in an IL-2 receptor that causes severe immunodeficiency (Figure 11.4). The gene therapy was highly successful, but caused two cases of acute lymphatic leukemia (ALL). In this instance, ALL was initiated by the insertional-mediated over-expression of LMO2, a bridging molecule in transcription complexes (see Table 11.3), which normally is expressed mainly in hematopoietic cell precursors. If inappropriately expressed, LMO2 blocks differentiation into mature T cells and acts as an oncogene, leading to over-production of undifferentiated lymphocytes and leukemia.

Acute transformation by viral oncogenes

The acute transforming retroviruses account for only a small proportion of naturally occurring tumors caused by retroviruses and they have never been isolated from humans. Several properties make them uncommon in nature, such as the requirement for a helper virus and the acute nature of the malignancies that they cause, which reduces the probability of horizontal transmission. However, the study of these viruses has played a very important role in the elucidation of viral oncogenesis. Most of the acute transforming viruses have two defining characteristics.

First, their genomes contain a viral *oncogene (v-onc)*, which is a gene that possesses specific transforming activity, often at a high level. It appears that most, perhaps all, viral oncogenes are derived from *proto-oncogenes (c-onc)*, normal genes that can influence cell growth. These are often the very same proto-oncogenes whose upregulation by non-acute oncogenic retroviruses leads to transformation. 'Proto-oncogenes' are so named because they are the ancestors of viral oncogenes that were 'captured'

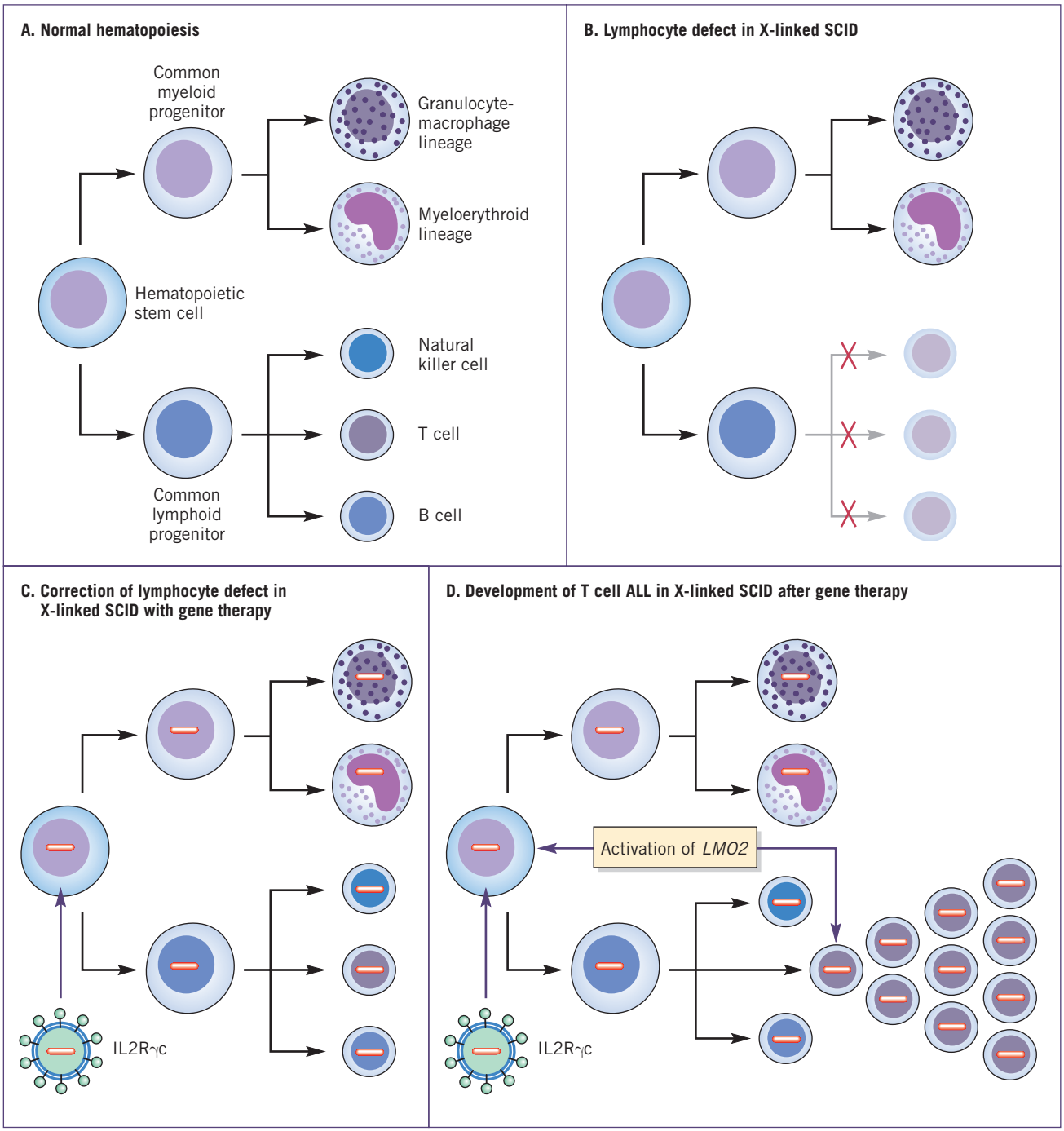

FIGURE 11.4 Retroviral insertional mutagenesis can occur in humans, as illustrated by acute lymphatic leukemia (ALL) as an unwanted complication of retroviral-mediated gene therapy. Panel A: normal hematopoiesis involves differentiation of pluripotential hematopoietic stem cells expressing the CD34 surface protein into two main committed progenitors: common myeloid and common lymphoid progenitors. Common myeloid progenitors can differentiate into all cells of the myeloerythroid lineage (granulocytes, macrophages, megakaryocytes, erythroid cells) and common lymphoid progenitors can differentiate into T cells, B cells and natural killer cells. Panel B: boys with X-linked SCID are deficient in the common γ chain of the interleukin-2 receptor (IL2Rγc), causing failure of normal lymphopoiesis. Panel C: gene therapy was used to correct the defect in X-linked SCID. CD34+ hematopoietic stem cells were infected ex vivo with a retrovirus encoding IL2Rγc and transduced autologous cells were reimplanted. Since the hematopoietic stem cells are progenitors of all hematopoietic cells, the transduced cells contribute cells carrying the integrated IL2Rγc DNA to all lineages, including the lymphocyte lineage, thereby correcting the immunodeficiency. The proportion of cells carrying the inserted retrovirus in the lymphoid lineages far outweighs that in other lineages. Panel D: unfortunately, T cell leukemia developed in two of the patients successfully treated in this way. In both children, the T cell acute lymphoblastic leukemia (T cell ALL) arose in cells in which the retroviral integration site was within or very close to the T cell oncogene *LMO2*. After McCormack MP, Rabbitts TH. Activation of the T-cell oncogene *LMO2* after gene therapy for X-linked severe combined immunodeficiency. *New England Journal of Medicine* 2004, 350: 913–922.

Category of oncogene (*example*)	Function of c-onc	Alteration in v-onc relative to c-onc	Action of v-onc
PTK protein tyrosine kinase (*src*)	Tyrosine phosphorylation activates intracellular signaling proteins	Loss of C terminal tyrosine or loss of receptor binding domain	Increased tyrosine phosphorylation causes sustained intracellular signaling
Serine-threonine kinase (*mos*)	Phosphorylation activates cell cycle proteins	Deletion of N terminal regulatory sequences	Increased phosphorylation activates cell cycle
G-protein (*ras*)	Cycles between GTP/GDP forms regulating intracellular signaling	Change in amino acid at codon 12	High GTP/GDP ratio causes sustained activation signals
Transcription factor (*myb*)	Interactions with transcription complex to enhance or reduce transcription	Deletion of regulatory domains or increase of mRNA or protein accumulation	Increased transcription of growth factors or cell cycle genes or reduction of anti-oncogene expression

TABLE 11.3 Some of the more common categories of oncogenes associated with acute transforming retroviruses
Abbreviations: c-onc: cellular proto-oncogene; *mos*: Moloney mouse sarcoma; *myb*:myeloblastosis; *ras*: rat sarcoma; *src*: sarcoma; v-onc: viral oncogene.

by recombination between retroviruses and cellular sequences and subsequently evolved by mutation.

Second, acute transforming viruses are, with some exceptions, defective due to the loss of part of their genome during their putative ancestral recombination event. Usually, the oncogene is located in the *env*, *pol* and/or *gag* region of the genome, so that the virus cannot encode the SU, TM, or some gag proteins (see Figure 10.2). These proteins are required to produce infectious virions and their absence renders such viruses defective and able to grow only in the presence of a replication competent non-transforming 'helper' retrovirus that encodes the *env* (or other missing) proteins in which the transforming virus genome is packaged. Figure 11.5 shows the genomic organization of a few representative acute transforming viruses.

Study of many acute transforming virus isolates has led to a catalog of the oncogenes that they carry, which has produced an explosion of information regarding the normal control of cell growth and division. Table 11.3 summarizes some of the more common viral oncogenes.

Acute oncogenic retroviruses transform cells in culture rapidly and are, like most oncogenic retroviruses, non-cytocidal. The rapid transforming effect reflects the fact that the oncogenes encoded by these viruses have evolved from their ancestral cellular genes in such a manner that they are relatively unresponsive to the checks and balances that control normal cellular growth factors. Oncogenes may escape control because they are transcribed and translated at a very high rate under the influence of the viral LTRs with their potent promoters and enhancers. Also, viral oncoproteins are often modified from their cellular counterparts by the loss or change in domains involved in downregulation by other cellular proteins. Based on their rapid transforming activity, these viruses may be quantified by 'focus forming' assays that are similar in principle to the plaque assays used to count lytic viruses.

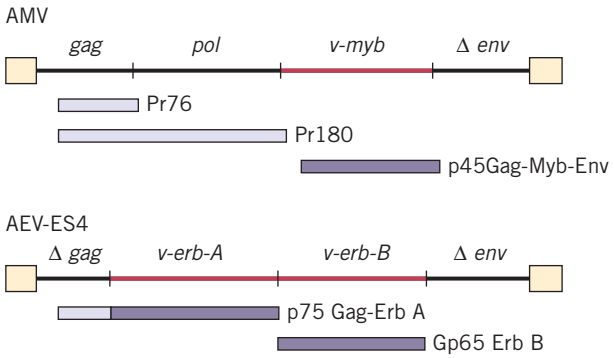

FIGURE 11.5 The genomic organization and major proteins encoded by two representative acute transforming retroviruses. The genome is shown above and the mRNAs representing the proteins are shown below, with the oncoproteins in dark purple. In some cases the oncoproteins are fused with viral proteins and in all instances they are modified from their cellular counterparts (not indicated in this figure). AMV: avian myeloblastosis virus; AEV: avian erythroblastosis virus: erb: erythroblastosis; myb: myeloblastosis; Δ*env*: truncated *env* gene.
After Coffin JM, Hughes SH, Varmus HE (eds). *Retroviruses*, Cold Spring Harbor Press, 1997, with permission.

In vivo, the acute transforming retroviruses cause tumors in weeks rather than months to years. This reflects their high transforming activity, such that further mutations may not be necessary for the production of tumors. Because cells are transformed rapidly and with a high probability, the tumors that result are usually polyclonal. Another consequence of the aggressive transforming activity is that some acute viruses may cause tumors in the absence of helper viruses, i.e. without replicating in the host.

In summary, the salient features of the acute transforming retroviruses (see Table 11.2) are the defectiveness of the virus requiring a helper virus for propagation, transformation by a viral oncogene, a short incubation period and the polyclonal nature of the transformed cells.

Trans-activation by viral accessory genes

Human T cell leukemia (HTLV-I) and bovine leukemia virus (BLV) comprise a relatively rare group of retroviruses whose oncogenic activity is due to a mechanism distinct from that used by the non-acute and acute transforming retroviruses. These viruses encode several accessory genes – particularly the *tax* gene – that are not only essential for their replication but also play a role in their transforming activity. However, the mechanism of oncogenesis is complex, since it appears that tax is necessary but not sufficient for oncogenesis and subsequent non-viral genetic events are required to produce tumors. HTLV-1, the best studied example, is described later in this chapter.

Oncogenic viral proteins

Rarely, a retroviral protein may have oncogenic activity. One example is pulmonary adenomatosis (jaagsiekte) of sheep, which is caused by an ovine retrovirus. It appears that the envelope protein of the virus can, by itself, activate an intracellular signaling pathway. This causes transformation of bronchoalveolar epithelial cells, which can eventually develop into adenocarcinomas of the lung. Oncogenic activity has been mapped to the transmembrane and cytoplasmic domains of the env protein, which activate the phosphoinositide-3-OH (PI3K)/Akt pathway.

Common themes

Certain common themes are present in all of the transforming retroviruses. Tumor formation is generally a multistep process that involves steps initiated by the virus and subsequent steps that are not virus-mediated. Often, tumor induction requires activation of more than one cellular oncogene. In addition to the over-expression of oncogenes, downregulation of cellular tumor suppressor genes frequently plays a role in the multistep pathway to tumor induction. Each oncogenic virus tends to produce a characteristic narrow range of tumors, related to the cells that it infects and the cell-specific activity of its LTR.

ONCOGENIC RETROVIRUSES: EXAMPLES

Murine leukemia viruses (MuLV), non-acute transforming retroviruses

Murine leukemia viruses were originally isolated from laboratory or wild mice and have been studied in more detail than any other group of oncogenic retroviruses. MuLVs can be classified into several groups including ecotropic, xenotropic, amphotropic and polytropic or mink-cell-focus-forming (MCF) viruses (Table 11.4). Ecotropic viruses will infect only murine cells, xenotropic viruses will infect cells from rats and other species but not mice and amphotropic viruses will infect cells from both mice and other species. MCF viruses do not exist as exogenous viruses, rather, they arise from endogenous sequences present in the mouse genome that can recombine with exogenous replication competent MuLVs. Each of these four classes of MuLVs is distinguished by having a different cellular receptor and cells infected with one class of viruses resist superinfection with other viruses of the same class because of receptor blockade (see Figure 4.5). Some receptors have been isolated while others are yet to be defined.

Mice, like other higher vertebrates, have endogenous retroviral sequences in their genomes. Presumably, these sequences originated when a retrovirus infected a germ cell and the provirus was then incorporated into the germline. Most laboratory mice have about 1000 copies of retrovirus sequences scattered throughout their genome. About 50 of these represent the MuLV family of retroviruses. Endogenous retroviral sequences usually are defective, containing only part of the viral genome, which

Class of MuLVs	Host species	Cellular receptor	Exogenous	Endogenous (number)	Source
Ecotropic	Mice	Cationic amino acid transporter (CAT-1)	Yes	Yes (0–3)	Laboratory mice
Xenotropic	Rats, other	Not known	No	Yes (~25)	Laboratory mice
Amphotropic	Murine, other	Phosphate transporter (Pit2)	Yes	No	Wild mice
Polytropic (MCF)	Murine, other	Rmc1	No	Yes (~25)	Laboratory mice

TABLE 11.4 Major classes of murine leukemia viruses (MuLVs). Each class utilizes a different cellular receptor, causing interference between individual viruses within each class but not between classes. Exogenous viruses exist as replication competent viruses that are transmitted horizontally in mice, while endogenous viruses exist as germline sequences (the number of copies per mouse genome is indicated) that are usually not replication competent but may recombine with exogenous viruses during the course of infection. Ecotropic viruses fall into two groups based on sharing of antigenic determinants, the FMR (Friend, Maloney, Rauscher) group and the Gross group (Gross and AKR viruses). Polytropic viruses are also called MCFV because they form transformed foci in mink cells. Except for the xenotropic group, members of all groups are oncogenic, producing tumors of T lymphocytes and, in a few instances, of other hematopoietic cells.

frequently includes the *env* gene. Although the endogenous sequences rarely give rise to replication competent viruses, some of them can recombine with each other or with exogenous replication competent murine retroviruses. Recombination is thought to occur when RNA transcripts of endogenous viruses and exogenous viruses are co-packaged in a virion. During the next round of replication, reverse transcriptase can use both the exogenous and the endogenous RNA during DNA synthesis. Viral recombinants play a significant role in the production of leukemia following infection with certain of the non-acute oncogenic retroviruses.

Leukemogenesis by MuLV is a complex multistep process. One major step is activation of proto-oncogenes by insertional mutagenesis (see Figure 11.3). From the analysis of many leukemic mice, a number of different proto-oncogenes have been shown to be activated in different cancers. Many of these are related to the viral oncogenes encoded by the acute retroviruses (see Table 11.3) and include proteins involved in transcriptional regulation, tumor suppressors such as p53, cyclins and cytokine receptors. It has long been recognized that different MuLVs tend to cause different types of leukemia depending upon the transformed cell (lymphocytes, erythrocytes, or other hematopoietic cell types). One major determinant of cellular specificity is the LTRs of different viruses, each of which is most active in certain types of cells. These specificities have been mapped to the enhancer sequences in some instances, based on experiments showing that engineered chimeric viruses exhibit the specificity of the virus that donated the enhancer sequence (Table 11.5).

Polytropic MCF viruses also play an important role in leukemogenesis induced by ecotropic MuLVs. Recombinant ecotropic viruses containing sequences (often in the *env* region) derived from MCF viruses appear concomitant with the development of leukemia and their sequences are frequently found in leukemic clones. It is thought that MCF recombinants may potentiate leukemogenesis because they use a different receptor, which permits dual infection of individual cells, thereby increasing viral load and raising the probability of insertional mutagenesis. In addition, certain MCF envelopes can bind to the erythopoietin receptor releasing the receptor from dependence on erythropoietin or Il-2 so that it is constitutively upregulated, creating autocrine stimulation.

Based on a variety of complex experiments, a conjectural scheme of leukemogenesis has been developed (Figure 11.6). Initial infection of the bone marrow with

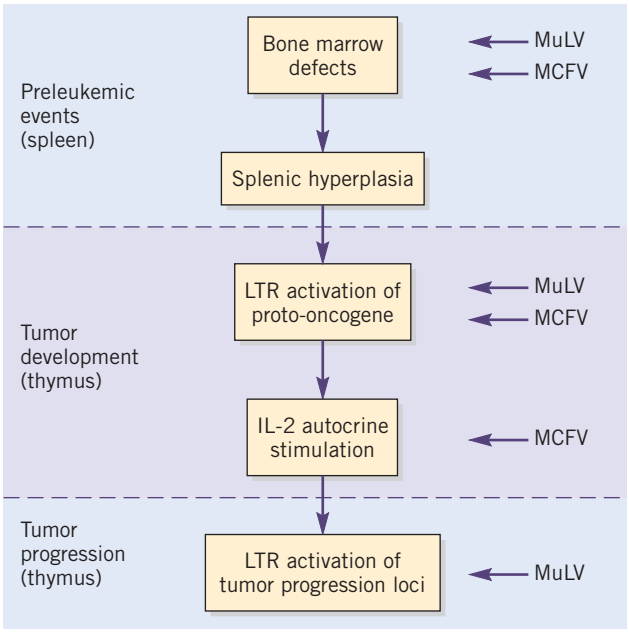

FIGURE 11.6 Multistep nature of leukemogenesis in mice infected with Moloney murine leukemia virus (M-MuLV). MuLV acts by insertional mutagenesis to upregulate many different proto-oncogenes that can act at several points in the development of leukemic clones. In addition, a second virus, the polytropic MCFV (mink-cell-focus-forming virus) is produced by the recombination of endogenous retroviral sequences with the replication competent MuLV. MCFV increases viral load and upregulates selected receptors for growth factors, complementing the activity of MuLV. After Fan H. Murine leukemia virus, in Ahmed R, Chen I (eds), *Persistent viral infections*, Wiley, New York, 1999, with permission.

MuLV strain	Genome backbone	Enhancer U3 region	Number of mice with indicated diagnosis		
			Erythro leukemia	Lymphoblastic leukemia	Myelogenous leukemia
Moloney	Moloney	Moloney	0	15	0
Friend	Friend	Friend	50	0	0
Moloney/Friend	Moloney	Friend	53	1	3
Friend/Moloney	Friend	Moloney	1	19	0

TABLE 11.5 The LTR of MuLV is an important determinant of the type of neoplasm produced by this group of oncogenic retroviruses. Moloney MuLV produces T cell lymphomas while the Friend MuLV causes erythroleukemias. In this experiment, the two virus strains were compared with chimeric viruses in which the U3 (unique 3') region of the genome of one virus was substituted into the backbone of the other virus. After infection of mice, the type of neoplasm co-segregated with the viral origin of the U3 region. The U3 domain of the viral LTR contains enhancer sequences and the tumor specificity was attributed to this part of the genome. Presumably the U3 region binds cellular proteins that differ between cells of lymphopoietic and erythropoietic lineages
Chatis PA, Holland CA, Silver JE *et al.* A 3' end fragment encompassing the transcriptional enhancers of nondefective Friend virus confers erythrogenicity on Moloney leukemia virus. *Journal of Virology* 1984, 52: 248–254, with permission.

MuLV induces transformation of multiple cell lines leading to hyperplasia of the spleen. MCF recombinant viruses are produced and play a significant role in these early events, in part by increasing viral load in infected cells. Some of these hyperplastic cell lines are further transformed into tumor cells by insertional mutagenesis that activates additional proto-oncogenes or inactivates tumor suppressor genes, enhanced by autocrine stimulation via cytokine receptors such as the erythropoietin receptor. Further activation of proto-oncogenes leads to tumor progression.

ASV, the prototypic acute transforming retrovirus

The avian sarcoma and leukosis viruses (ASLV) are a group of oncogenic retroviruses that include both acute and non-acute transforming viruses. The ALV group mainly causes leukemias (leukosis), tumors of hematopoietic cells (lymphocytes, myelocytes, erythrocytes or their precursors), while the ASV group mainly causes fibrosarcomas. The ASV group includes Rous sarcoma virus (RSV) strains – all of which carry the *src* gene – and other avian sarcoma viruses that carry viral oncogenes other than *src*, such as *fps*, *ros* or *yes*. The ASLVs were the first retroviruses isolated and have served as prototypes to work out many of the basic biological and molecular features of retroviruses and establish new paradigms in virology and oncology. Rous sarcoma virus (RSV) was one of the first viruses shown to be a filterable agent (Peyton Rous, 1910) capable of causing a transmissible sarcoma in chickens and investigations of RSV led to development and proof of the hypothesis of reverse transcription (Temin, 1970) and to the discovery of the relationship between viral oncogenes and cellular proto-oncogenes (Bishop and Varmus, 1976; see Sidebar 1.2). This account will focus on RSV.

Replication competent and defective viruses

A number of isolates of RSV, from chickens with sarcomas, have been characterized. All of these viruses carry the *src* oncogene (also called 'v-src'), are acutely transforming in chick embryo cultures and induce sarcomas under appropriate experimental conditions. Some of these viruses are replication competent because they have an expanded genome that accommodates a full complement of retroviral genes in addition to the *src* gene.

Other isolates of RSV are defective in their *env* genes and can only replicate in cells that are co-infected with a replication competent helper virus that encodes a functional *env* gene (see Figure 11.2). The helper viruses are all members of the ALV group and stocks of defective RSVs must be propagated together with an ALV isolate. ALVs, although they cannot transform fibroblasts, can cause leukosis in chickens. ALV isolates differ in their envelopes and can be classified into subgroups depending upon the receptor specificity of the envelope. Since different ALVs can be used to complement a given defective RSV isolate, that isolate can exhibit different subgroup identities depending upon the ALV virus that serves as helper. Historically, when it was discovered that some stocks of RSV contained an associated virus that was required for propagation of the sarcoma virus, the term Rous-associated virus (RAV) was introduced; subsequently RAV was recognized to be ALV that acted as a helper for the defective transforming RSV.

HTLV-I, a trans-acting oncogenic retrovirus

Human T cell leukemia virus type I (HTLV-I) is an exogenous virus of humans that causes adult T cell leukemia (ATL) as well as a neurological disease (HAM, HTLV-associated myelopathy, also called tropical spastic paraparesis) that is not relevant to the present discussion. HTLV-I is transmitted from person-to-person in several ways, via breast feeding, sexual contact, or blood (transfusions, blood products and contaminated needles). HTLV-I and ATL have an unusual geographic pattern, being endemic in a few selected areas, such as Japan and the Caribbean islands, but relatively rare in most regions of the world. HTLV-I infections are usually asymptomatic and only 1–5% of infected persons ever develop the disease after an incubation period that can be as long as 20–30 years. Acute ATL is characterized by circulating malignant CD4+ T cells, which infiltrate skin and viscera, usually causing death within one year. There are a few other related oncogenic retroviruses, such as BLV (bovine leukemia virus) which produces B cell leukemia.

The mechanism of oncogenesis for HTLV-I is only partially understood. These viruses do not carry established oncogenes like the acutely transforming retroviruses and there is no evidence of upregulation of a cellular proto-oncogene. The *tax* gene of HTLV-I, which transactivates the viral LTR (similar to the action of *tat* in HIV) and is required for viral replication, plays an essential role in transformation.

Several observations are relevant to the mechanism of oncogenesis:

1. HTLV-I preferentially infects CD4-positive T lymphocytes, likely due to post-entry selection bottlenecks; tropism is not related to its receptor, a glucose transporter (GLUT1) which is expressed on many cell types. The provirus is integrated randomly into the genome of the host cell, followed by viral replication that requires the action of *tax* and *rex* gene products. The virus is non-cytocidal and persists in infected cultures but remains highly cell-associated so that it is most readily transmitted by co-cultivation of infected with uninfected cells.
2. HTLV-I can immortalize primary T cell cultures, producing CD4+ cell lines that can grow continuously in the absence of Il-2 (exogenous Il-2 is required to maintain cultures of primary T cells). However, transformation occurs in only a small fraction of infected T cells and the cultures evolve slowly through a polyclonal to a monoclonal phase, suggesting that additional events are required to produce the transformed phenotype.
3. Tax transforms immortalized rodent cell lines, converting them into cells that will form colonies in agar and produce tumors in immunosuppressed mice.

4. ATL is oligoclonal and cell lines cloned from patients contain HTLV-I proviruses, but these are often highly deleted sequences that are incapable of replicating. Also, there is little transcription or translation of the *tax* gene in ATL cell lines.

The Tax protein acts via mechanisms that are similar to those used by oncogenic DNA viruses (see Chapter 12). Tax is highly pleiotropic and the following description is only a partial description of its action. Tax is a nuclear phosphoprotein that is transported into the cytoplasm, where it perturbs many signaling pathways. One salient effect is to activate the cell cycle by interfering with the action of p53, pRb (the retinoblastoma protein) and other proteins that act as gatekeepers on the cell cycle. In addition, Tax leads to the inactivation of IκB, the cytoplasmic protein that binds NFκB, an important transactivator of transcription in lymphocytes. Inactivation of IκB frees NFκB to activate transcription in an uncontrolled manner. Specific effects of transactivation include the upregulation of cytokines (IL-2, IL-13 and IL-15) and cytokine receptors (IL-2Rα), which also drive lymphocytes into the cell cycle. Finally, Tax interferes with several cellular mechanisms that normally detect genetic damage and activate the apoptosis pathway, thus permitting genetically abnormal immortalized cells to survive and become transformed.

These observations have been used to construct a working hypothesis regarding the oncogenic activity of HTLV-I (Figure 11.7). Initially, the virus infects CD4+ T lymphocytes and the viral genome is integrated as a provirus. The Tax protein – through the transactivation of selected cellular genes – immortalizes T lymphocytes, which carry the viral genome for the life of the human host. In a small proportion of viral carriers, one or more additional low probability genetic events, such as chromosomal recombination, transform an occasional T cell line into an oncogenic clone. Initially, these clones produce smoldering ATL that develops into acute ATL if there is an outgrowth of a highly aggressive cell clone. During the genetic events that create highly oncogenic T cell clones, the HTLV-I provirus may be altered, 'inactivating' the *tax* gene, which is no longer required to maintain the transformed phenotype.

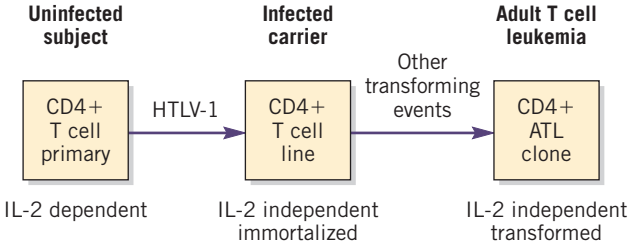

FIGURE 11.7 Diagram of putative transforming action of HTLV-I. It is postulated that ATL (adult T cell leukemia, the cancer associated with HTLV-I) is produced by a multistep sequence of events. The first event is infection of CD4+ T lymphocytes by HTLV-I which leads to integration of the provirus in the genome of the host cell. The action of the HTLV-I *tax* gene immortalizes clones of T lymphocytes. In a small proportion (<5%) of infected carriers, these cells undergo subsequent genetic events that convert them into leukemogenic clones.
After Kashanchi F, Pise-Masison C, Brady JN. Human T cell leukemia virus, in Ahmed R, Chen I (eds), *Persistent viral infections*, Wiley, New York, 1999, with permission.

BIOLOGY OF CANCER

Cellular specificity of tumors induced by oncogenic retroviruses

Each retrovirus tends to produce a narrow range of characteristic neoplasms in its natural host. This specificity is determined by a number of variables. First, most viruses utilize one or a very few cellular surface molecules as their receptors. Second, for retroviruses where the LTR plays a role in transformation, the activity of promoters and enhancers within the LTR is often greater in those cell types that are typical targets of each virus (see Table 11.5). Finally, the cellular environment may be a determinant in the further conversion of virus-transformed cells into malignant clones. Thus, T lymphocytes transformed by MuLVs apparently migrate to the thymus where they undergo further genetic changes that confer tumorigenic potential, thereby causing T cell leukemias or thymomas.

Multifactorial induction of cancer

Understanding of the molecular biology of cancer has evolved rapidly, to the point where a comprehensive set of mechanisms has been implicated in the induction of most neoplasms (Sidebar 11.1). All of these mechanisms involve the molecular biology of the cell and may be grouped into three major categories: lack of need for external growth signals and insensitivity to anti-growth signals; unlimited growth potential and evasion of apoptosis; tumor metastasis and sustained angiogenesis. This comprehensive view has important implications. First, it explains the low frequency and relatively long course to cancer. The multiple genetic changes required for cancer induction typically occur only in some members of a population over a prolonged interval. Second, it explains why an oncogenic virus may be necessary but not sufficient to produce neoplasia. The virus, through a variety

SIDEBAR 11.1

Molecular determinants in the conversion from normal to the malignant cellular phenotype

Growth signals
- Self sufficiency in growth signals
- Insensitivity to growth inhibitory signals

Cell division
- Limitless potential for cellular replication
- Escape from apoptosis

Oncogenesis
- Metastasis and tissue invasion
- Sustained angiogenesis

After Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000, 100: 57–70, with permission.

of molecular actions, can perturb some but not all of the cellular determinants that lead to neoplasia. The routes to tumorigenesis are undoubtedly diverse, both in the order of the changes that occur and in the number of mutations involved. In some instances, a single mutation may activate or inactivate an essential change in cellular phenotype, while in other instances several mutations may be required; conversely, some mutations may alter several critical phenotypes. Finally, an oncogenic virus may be associated with one or several of the mutations involved in the emergence of a given neoplasm.

RETROVIRUSES AS VEHICLES FOR GENE THERAPY

The mode of replication that confers oncogenic potential on retroviruses also makes them attractive vectors for gene therapy (Figure 11.8). A replication competent retrovirus may be 'engineered' to remove its structural genes and substitute a 'therapeutic' gene that is destined to be transduced into the stem cells of a recipient. In addition, it can be useful to insert a 'selectable' gene, whose gene product facilitates the selection of cells that have been transfected with the retrovirus vector. To introduce the therapeutic gene into target cells, the retrovirus vector must be packaged into virions. This is accomplished by transfecting the vector into 'packaging' cells that have been stably transduced to express the structural genes of a retrovirus. Transfected cells will produce retrovirus particles that contain the engineered vector and these particles can efficiently infect appropriate target cells. Figure 11.4 shows an example of the successful application of such a retrovirus vector and also highlights the potential complications of this gene therapy strategy.

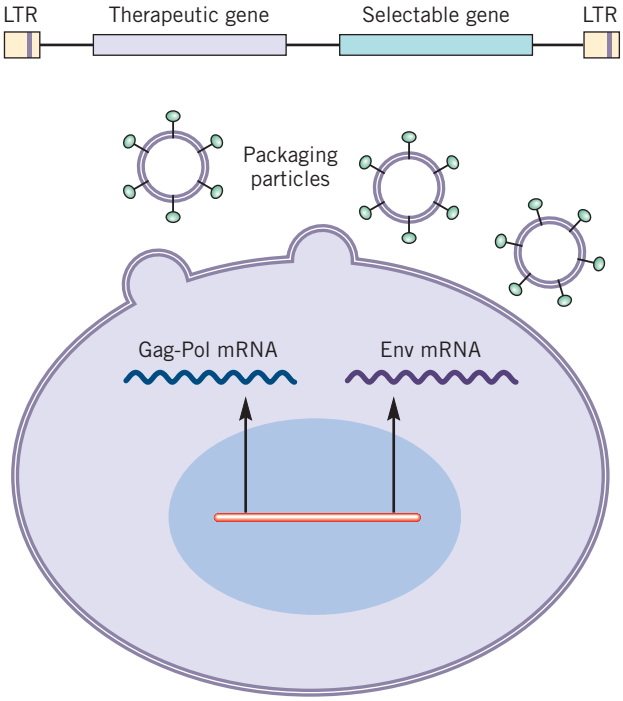

FIGURE 11.8 Retroviruses as vectors for gene therapy. Top: the proviral (DNA) form of a retrovirus that has been 'engineered' by removing its structural genes and inserting a 'therapeutic' gene and a 'selectable' gene. Bottom: a schematic view of a packaging cell that has been stably transduced to express the structural genes of a retrovirus. The structural proteins will self assemble to produce packaging particles (empty virions) lacking a genome. When transfected with the retrovirus vector, these cells will produce virions containing the vector genome. When such virions infect a target cell, the integrated provirus will express the therapeutic and selectable proteins. After Robinson HL, Rein A, Speck NA. Avian and murine retroviruses, in Nathanson N, Ahmed R, Holmes KV *et al.* (eds), *Viral pathogenesis*, Lippincott Raven Publishers, Philadelphia, 1997, 629–656, with permission.

REPRISE

The retroviruses are a family of viruses that is distinguished by their unusual mode of replication, which determines their mechanisms of oncogenesis. Retroviruses are plus-stranded RNA viruses containing a reverse transcriptase that converts them into a double-stranded DNA intermediate, which is integrated into the host cell genome and then transcribed back into mRNA and new RNA viral genomes. These viruses employ three distinct oncogenic strategies:

1. Non-acute transforming retroviruses act by insertional mutagenesis; they are found in several animal species but there are no human representatives of this virus class. The DNA provirus alters the activity of the host genome either by inactivating a tumor suppressor gene or by upregulating a host gene that initiates cell cycling via promoter or enhancer pathways.
2. Acutely transforming retroviruses are relatively rare and there are no human representatives. These retroviruses incorporate an oncogene that usually replaces one of their essential genes, so that they are replication-defective and require a replication-competent 'helper' virus. The viral oncogene (*v-onc*) is derived from a cellular counterpart (*c-onc*) but is not subject to normal cellular regulation so that it initiates uncontrolled cell cycling.
3. Oncogenic retroviruses with trans-activating accessory genes encode one or two additional viral genes that have the ability to immortalize host cells. This is a class of retroviruses with few members but there is at least one human example, HTLV-I.

Certain common themes are present in all of the transforming retroviruses. Foremost is aberrant expression of normal host genes. In addition to the over-expression of oncogenes, downregulation of cellular tumor suppressor genes frequently plays a role in tumor induction. Each oncogenic virus tends to produce a characteristic narrow range of tumors, related to the cells that it infects and the cell-specific activity of its long terminal repeat (LTR). Tumor formation is generally a multistep process that involves steps initiated by the virus and subsequent steps that are not virus-mediated.

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Chapter 12

Viral Oncogenesis: DNA Viruses

Neal Nathanson and Erle S. Robertson

CHAPTER CONTENTS

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REPRISE

FURTHER READING

INTRODUCTION

Many different families of DNA viruses have members that are oncogenic either in their natural hosts or in experimental animals (Table 12.1). The small oncogenic DNA viruses (adenoviruses, polyomaviruses and papillomaviruses) utilize mechanisms to induce neoplasms that are, to some extent, different from those used by the large oncogenic DNA viruses (such as some herpesviruses and poxviruses).

Small DNA viruses

Most of the small oncogenic DNA viruses can immortalize or transform cells in culture, although this potential is usually restricted to selected cell types and specific conditions. This capability is attributed to virus-encoded proteins that are sometimes dubbed oncoproteins. However, unlike the RNA viral oncogenes, they are not derived from cellular counterparts but serve essential functions for virus propagation, often by inducing S phase genes and extending the life of the infected cell. In turn, this can lead to the immortalization of the infected cell and eventual transformed state. These viral oncoproteins function by blocking the action of normal cellular factors, including tumor suppressor proteins that act as brakes on the cell cycle.

Large DNA viruses

Relatively few large DNA viruses are oncogenic and, for the most part, they do not encode identifiable 'oncogenes', nor do they act primarily by blocking cellular tumor suppressor proteins. However, it is now clear that the large DNA viruses have a number of accessory genes that can function as oncogenes with transforming potential. In many instances, these virus-encoded proteins immortalize cells by interacting with extracellular growth factors and the intracellular signaling systems that respond to growth factors, resulting in perpetual cellular division. In addition, they may encode homologs of cellular proteins that activate the cell cycle or interfere with intracellular signaling that leads to apoptosis. In some instances, they target the well known tumor suppressor proteins, p53 and the retinoblastoma protein (pRb).

See Chapter 11 for definitions of immortalization and transformation.

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Host	Virus class	Example (virus and *disease*)	Natural tumors in host of origin	Oncogenic mechanism(s)
Human	Adenoviridae	Adenovirus*	No Experimental only	Encodes transforming protein(s) Inactivates tumor suppressors
	Papillomaviridae	HPV* *Cervical carcinoma*	Yes	Encodes transforming protein(s) Inactivates tumor suppressors
	Hepadnaviridae	HBV *Hepatocellular carcinoma*	Yes	X protein Inhibits DNA repair Activates transcription Alters Ras signaling
	Herpesviridae	EBV* *Burkitt lymphoma*	Yes	Immortalizes cells Encodes transforming proteins
		HHV-8* *Kaposi's sarcoma*	Yes	Activates signaling pathways Inhibits p53 and Rb pathways
Animal	Polyomaviridae	MPV (mouse) SV40 (monkey)	No Experimental only	Encodes transforming protein(s) Inactivates tumor suppressors
	Papillomaviridae	CRPV (rabbit) *Papilloma*	Yes	Encodes transforming protein(s) Inactivates tumor suppressors
	Hepadnaviridae	WHV (woodchuck) *Hepatocellular carcinoma*	Yes	X protein Inhibits DNA repair
	Herpesviridae	MDV (chicken) *Marek's disease*	Yes	Immortalizes cells Encodes transforming protein
	Poxviridae	Myxoma (rabbit) *Papilloma*	Yes	Encodes growth altering proteins

TABLE 12.1 Representative oncogenic DNA viruses of animals and humans. The viruses described in this chapter are indicated with an asterisk (*)
Abbreviations: CRPV: cottontail rabbit papillomavirus; EBV: Epstein-Barr virus; HBV: hepatitis B virus; HHV8: human herpesvirus 8; HPV: human papillomavirus; MDV: Marek's disease virus; MPV: mouse polyomavirus; SV40: simian virus 40; WHV: woodchuck hepatitis virus.

MECHANISMS OF ONCOGENESIS

Tumor suppressor genes

Most of the proto-oncogenes described in Chapter 11 function in normal cells to promote cell growth. In addition, cells also encode a number of genes whose products act as negative regulators of cell growth. These genes are often called tumor suppressor genes because they are mutated or deleted at a high frequency in certain cancers. Together, the positive and negative regulators of cell growth constitute a complex system of checks and balances. The small DNA viruses produce proteins that bind and inactivate these tumor suppressor proteins (Table 12.2), thereby activating cell cycle and moving cells from G1 into S phase. Consequently, these viruses can initiate tumor formation.

	pRb cellular anti-oncoprotein	p53 cellular anti-oncoprotein
Virus	Viral protein (below) that complexes with cellular anti-oncoprotein (above)	
Simian virus 40	Large T antigen	Large T antigen
Human papillomavirus	E7	E6
Adenovirus	E1A	E1B
Human herpes virus 8	LANA	LANA
Epstein-Barr virus	EBNA 3C	

TABLE 12.2 DNA tumor viruses that encode proteins that inactivate cellular anti-oncogenes
pRb: retinoblastoma protein. After Brooks GF, Butel JS, Morse SA. *Jawetz, Melnick & Adelberg's medical microbiology*, 21st edn, Chapter 43, Tumor viruses and oncogenes, Appleton and Lange, Stamford, 1998, with permission.

pRb and p53 proteins

Two of the most important tumor suppressor genes are the retinoblastoma susceptibility (*Rb*) gene and the *p53* gene. The pRb protein controls the transition from S to G1 phase of the cell cycle (Figure 12.1). In the active, hypophosphorylated form, the Rb protein associates with the family of E2F transcription factors, sequestering them and abrogating their function, which is to promote the transition of cells from G1 to S phase. By this action, pRb acts as a brake on the cell cycle. Cells are moved into and through the cell cycle by the action of different cyclin-dependent kinases (CDKs), proteins that are activated by complexing with cyclins. One of the functions of some of

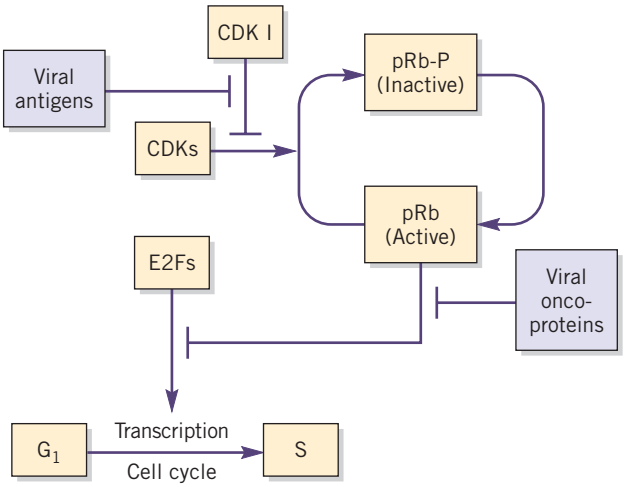

FIGURE 12.1 The action of the retinoblastoma protein (pRb) and the effect of viral oncoproteins that bind it. pRb cycles between an active (un-phosphorylated) and an inactive (phosphorylated) form, catalysed by cyclin-dependent kinases (CDKs). The active form (pRb) binds several transcription factors (E2Fs) that are required to recruit cells from G1 to S phase of the cycle and sequestration of these factors acts as a brake on cell division. Several viral oncoproteins (E7 of HPV; E1A of adenovirus; large T antigen of SV40) bind and sequester pRb, abrogating its braking action and promoting cellular proliferation and tumorigenesis. In addition, certain viral antigens inhibit the action of proteins that block CDKs (CDKI), thereby inactivating the Rb protein and releasing a block on the cell cycle.

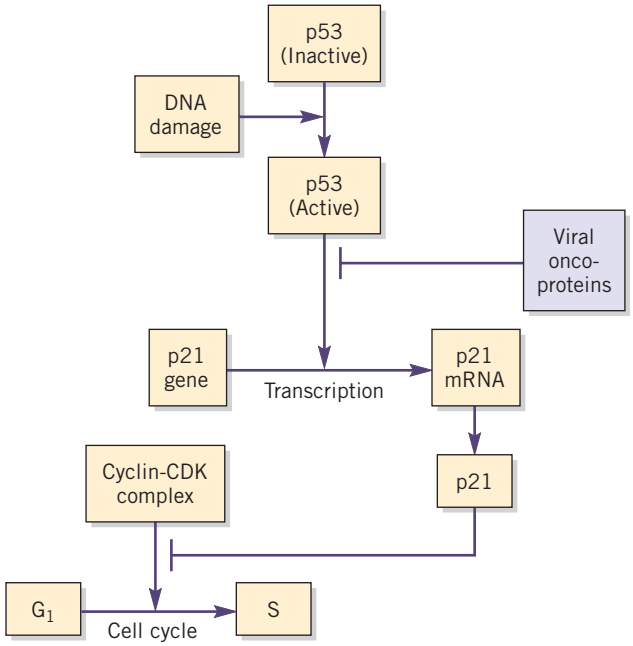

FIGURE 12.2 The action of the p53 protein and the effect of viral oncoproteins that block its action. p53 accumulates and is activated by damage to cellular DNA or aberrant growth signals and functions to protect multicellular organisms against the growth or survival of such damaged cells. One action of p53, as illustrated in this figure, is to cause growth arrest by initiating the transcription of a protein, p21, which binds to a complex of cyclin-CDK (cyclin-dependent kinase). This complex activates the cell cycle by moving cells from G1 into S phase. When p21 binds this complex it is inactivated, providing a brake on the cell cycle. DNA viral oncoproteins (HPV E6, adenovirus E1B, SV40 large T antigen) bind p53 and interfere with p53-mediated growth arrest, thus removing a critical brake on the cell cycle and contributing to the transformation or immortalization of cells.

the CDKs is to inactivate the pRb protein by phosphorylation. Highly phosphorylated pRb no longer binds E2Fs, the responsive genes are then derepressed, removing the braking action of pRb on the cell cycle.

p53 is a key tumor suppressor protein. Alteration or inactivation of p53 is a frequent component of the multistep process that leads to oncogenesis (Figure 12.2). Over 50% of a variety of human tumors exhibit mutations in the p53 gene. The importance of p53 is further substantiated by the observation that knockout mice lacking functional p53 develop normally but are prone to tumor development.

p53 acts to repair or delete abnormal cells that would have a deleterious effect on the organism. The concentration of p53 increases in response to DNA damage and induces growth inhibitory genes, DNA repair genes or genes that promote apoptosis (programmed cell death). p53 acts as a transcription factor, inducing expression of a number of proteins including p21. p21 binds and inhibits certain CDKs, thereby providing a brake on the cell cycle. Alternatively, extensive DNA damage can result in p53-mediated apoptosis by inducing pro-apoptotic proteins, such as Bax (see Chapter 4), which trigger the caspase cascade, a sequence of protein activations leading to proteolysis of selected proteins essential for viability of the cell.

Both growth arrest and apoptosis initiated by p53 are blocked by a number of oncoproteins encoded by DNA viruses. Some viral oncoproteins interfere with effector domains of p53 while others enhance its ubiquitination and degradation. Viral oncoproteins may also act downstream of p53 by binding and inactivating Bax or possibly by elevating cyclins that then neutralize p21 activity.

There is interaction between the pRb and p53 pathways. The inactivation of pRb by aberrant growth signals can activate $p14^{ARF}$, an E2F1 responsive gene. This protein stabilizes p53 by antagonizing the mdm2 protein which targets p53 for degradation. In turn, this leads to apoptosis, circumventing pRb inactivation.

SELECTED EXAMPLES OF DNA TUMOR VIRUSES

To illustrate the mechanisms of oncogenesis by DNA tumor viruses, four examples will be discussed (see Table 12.1). Three of these, human papillomaviruses (HPVs), Epstein-Barr virus (EBV) and Kaposi's sarcoma herpesvirus (HHV8), are significant causes of cancer in humans, while the fourth, human adenovirus, has served as an important model for the study of oncogenes and has been used as a vector for human gene therapy. These four viruses also illustrate the differences between the small DNA viruses (papillomavirus and adenovirus) and the large DNA viruses (EBV, HHV8).

Papillomaviruses: HPV and cervical carcinoma

Human papillomaviruses (HPVs) are a large family of ubiquitous small DNA viruses that are distinguished by their tropism for epithelial cells at different body

HPV type	Anatomic site	Skin or mucosal disease	Risk of cancer
1, 4	Sole, palm	Plantar warts	None
2, 57	Skin, genital mucosa	Common warts	None
3, 10	Skin, genital mucosa	Flat warts	None
6, 11	Anogenital area, larynx	Warts	$\ll$1%
16, 18, 31, 45	Cervix, anogenital area, esophagus	Condylomas, dysplasias, carcinomas	1–3%
5, 8, 47	Skin, esophagus	Epidermodysplasia verruciformis	30–40%

TABLE 12.3 Some of the more common types of human papillomavirus (HPV) and the diseases they cause
After Chow LT, Broker TR. Small DNA tumor viruses, in Nathanson N, Ahmed R, Gonzalez-Scarano F *et al.* (eds), *Viral pathogenesis*, Lippincott Raven Publishers, Philadelphia, 1997, 267–301, with permission.

sites. Based on molecular differences (>10% sequence diversity), over 100 different types of HPVs have been identified by cloning and sequencing. One of the attributes of HPV is the striking differences in pathogenicity of these different types. HPVs can be sorted into 5–10 distinct groups based on their localization to different cutaneous and mucosal surfaces of the body. All of these viruses cause benign epithelial overgrowths (variously called warts, papillomas and condylomas). Under normal circumstances, immune surveillance keeps the viruses under control, such that infections are either latent or regress spontaneously. However, some infections can persist and some virus types can also cause (at a low frequency) malignant tumors, notably oral, cervical and penile cancers (Table 12.3).

Initially, HPV infects basal cells in the epidermis through a wound in the epithelium. As the wound heals and the epithelium is re-established, the cells at the lower epidermal strata divide and some of the daughter cells move toward the surface of the skin while undergoing differentiation. The virus undergoes sequential steps in its replicative cycle in the differentiating epithelium; progeny virions are found exclusively in some of the superficial dying and dead cells (Figure 12.3). This dependence upon squamous cell differentiation has made it impossible to grow and study HPV in conventional cell cultures, impeding research for many years. However, HPVs will replicate in special keratinocyte rafts or organotypic cultures.

The HPV genome and its expression

The HPV genome is divided into three regions, the early (E) and late (L) genes and a non-coding transcription regulatory region which overlaps the origin of replication. Early genes mainly encode factors required for transcription and replication of the viral genome, while the late genes encode the virion structural proteins.

The transcription profile of HPV mRNAs and physical states of the HPV DNA differ in benign papillomas and condylomas (warts) from that seen in high grade dysplasias and carcinomas. In papillomas, condylomas and low grade dysplasias, viral DNA is detected as non-integrated nuclear episomes, together with varying levels of mRNAs representing different viral genes, of which the E4 and E5 messages are most abundant. There is great heterogeneity in the amounts of viral DNA and RNAs in different cells within any papillomatous lesion or among lesions from different patients. Viral capsid proteins may be visualized in some of the differentiated cells.

In contrast, in *carcinoma in situ* and invasive cancers, infectious virus is no longer produced. Rather, viral DNA is often integrated into the host chromosomes, accompanied by some loss of viral sequences. The viral DNA copy number is significantly reduced relative to that in productive infections, as it no longer can amplify. Furthermore, integration disrupts the viral transcription unit and most HPV genes are not expressed. However, the E6 and E7 mRNAs are present at elevated levels in these cancerous lesions and are not detected in the adjacent, histologically normal tissues. In addition, viral DNA integration appears to destabilize DNA and contributes to the chromosomal abnormalities associated with HPV-transformed cells. These observations, together with the properties of the E6 and E7 proteins, constitute compelling evidence that HPV can cause cancers in humans.

The HPV receptor α6 integrin complexes with either β1 or β4 integrin subunits and appears to be expressed in many cell types and species. This suggests that the exquisite tissue specificity cannot be attributed to receptor distribution. The intimate relationship between productive infection and epithelial differentiation probably reflects the requirement for certain combinations of cellular transcription factors that are only supplied by epithelial cells at sequential stages of squamous differentiation (Figure 12.3). By using the keratinocyte raft culture system, the activities of the promoters responsible for the expression of the viral oncogenes have been investigated. They are regulated by cis elements that activate transcription in the differentiated cells, by cis elements that repress transcription in the undifferentiated basal cells and by histone

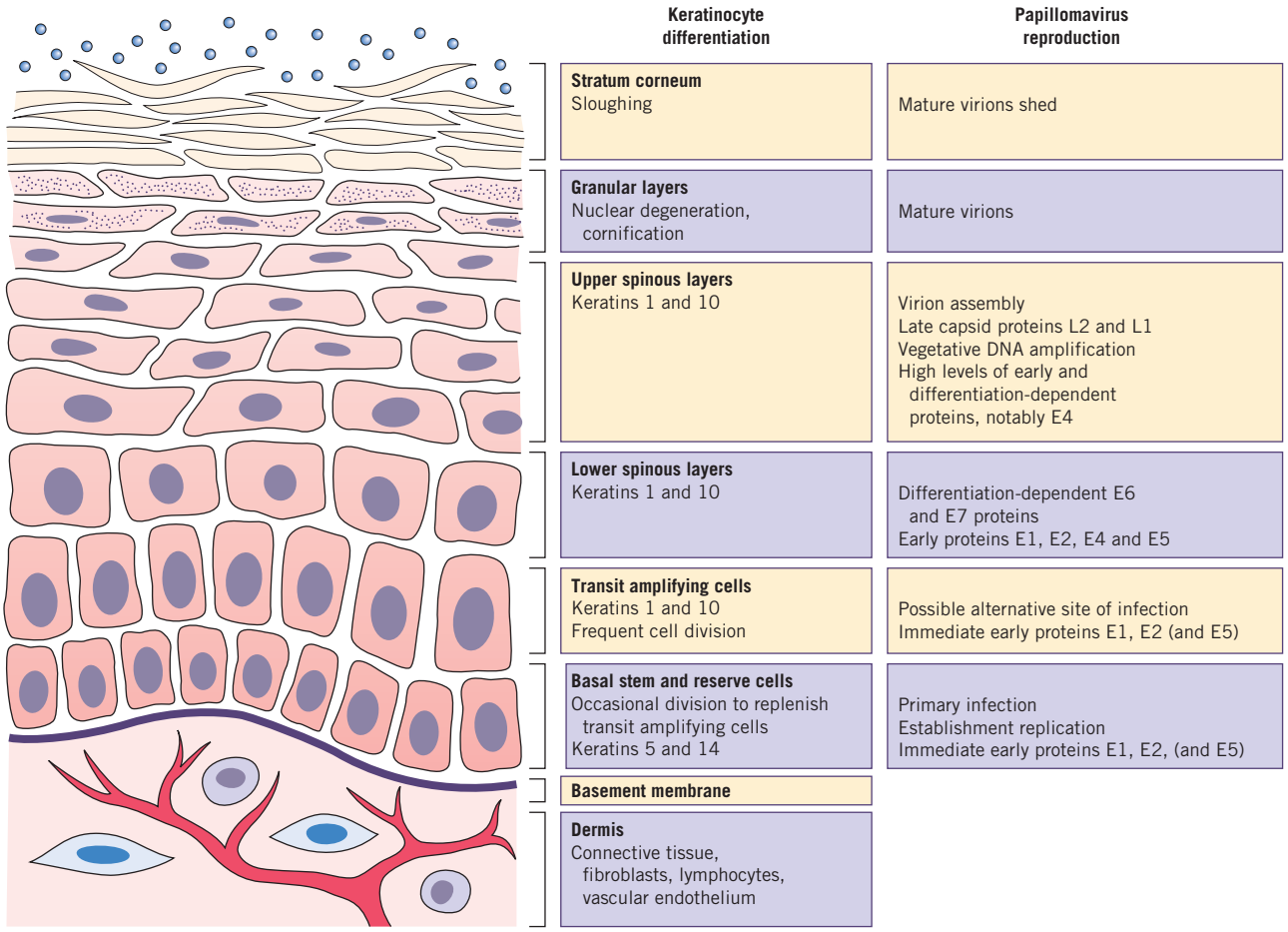

FIGURE 12.3 Sequence of transcriptional events in the synthesis of papillomavirus in different layers of the epidermis. This diagram shows the steps in generation and maturation of keratinocytes and the corresponding steps in the sequential cascade of viral replication. After Chow LT, Broker TR. Small DNA tumor viruses, in Nathanson N, Ahmed R, Gonzalez-Scarano F *et al.* (eds), *Viral pathogenesis*, Lippincott Raven Publishers, Philadelphia, 1997, 267–301, with permission.

deacetylases that remodel chromatin and downregulate transcription in the basal cells.

In addition to virus-encoded proteins, viral DNA replication also depends on the host cells to supply cellular replication proteins such as DNA polymerases, topoisomerases and enzymes that synthesize the deoxyribonucleoside triphosphate substrates. Most of these cellular proteins are normally present in S phase cells but not in post-mitotic, differentiated cells. This explains the need for viral oncoproteins that reactivate S phase genes in these cells. It also accounts for the oncogenicity of certain HPV types when these viral oncogenes are inappropriately upregulated in the normally quiescent stem cells. The transforming ability of HPV can be mapped to three of the early proteins, E5, E6 and E7, all of which are also required for productive virus replication.

E5 protein

The E5 protein appears to act by its influence on signaling through the EGF (epidermal growth factor) pathway. The EGF receptor is constantly recycled (like other receptors) by internalization into endosomes from which some molecules return to the cell surface while others are degraded. E5 associates with the EGF receptor and increases the proportion of molecules that are returned to the cell surface, thereby enhancing the concentration of receptor on the cell surface. The result is expansion of the infected cell population.

E6 and E7 proteins

The E6 and E7 genes are required for productive infection with HPV (see Figure 12.3). E7 binds to and inactivates the retinoblastoma (pRb) and related proteins, thereby overcoming the cell-cycle brake (see Figure 12.1). For certain HPV types, such as the oncogenic HPV-18, expression of the E7 protein in differentiated keratinocytes is sufficient to promote S phase re-entry in a fraction of differentiated keratinocytes, thus providing a milieu conducive for viral DNA replication.

The role of E6 in differentiated cells is partially understood. It cannot promote S phase entry in post-mitotic differentiated cells, but it may lengthen the viral reproductive phase. One of the better understood properties of E6 is its ability to inactivate the p53 protein by targeting p53 to the ubiquitin-mediated proteosome pathway (see Figure 12.2). In addition, E6 activates telomerase, an enzyme necessary to maintain the telomere lengths of chromosomes, critical for escaping senescence and

sustaining long-term cell proliferation. Finally, E6 can prolong the life of infected cells by binding Bak, a pro-apoptotic member of the Bcl-2 family (see Chapter 4).

The oncogenicity of the the E6 and E7 genes, when expressed in undifferentiated epithelial cells, has been demonstrated in two experimental systems, immortalization of primary keratinocytes in culture and tumor production in transgenic mice. E6 and E7 can extend the life span of primary cells, but immortalization usually requires mutations in additional cellular genes. Furthermore, transformed cells are not tumorigenic in nude mice and tumor formation requires additional cellular mutations. In HPV-immortalized cells, mutations can occur as a consequence of excessive cell cycling in the absence of a functional p53 to safeguard the integrity of the genome. Because these cells are already deficient in functional p53 protein, few HPV-associated cancers have mutated p53, at least until late stages, when the cellular genome is highly unstable. In contrast, cancers that are not associated with HPVs often exhibit mutations in the p53 gene.

In animal model systems, the effects of E6 and E7 depend upon the promoters that drive them, which determines the cell type in which the viral oncogenes are expressed, the level of transcription and nature of the tumors that are induced. Transgenic systems that mimic HPV-associated premalignant lesions and cancers, such as a keratin 14 promoter driving E6 or E7 from HPV-16, produce dysplasias or cancers of epithelial tissues because this promoter is active in basal and stem cells. On the other hand, when expressed from a differentiation-dependent promoter, such as the keratin 1 promoter, E6 and E7 of HPV-18 cause only papillomas.

Constitutive expression of E6 and E7 has different tumorigenic effects and the two proteins act synergistically in vivo (Figure 12.4). Transgenic mice expressing E7 develop epidermal hyperplasia suggesting that E7 plays a role in the early stages of carcinogenesis, while E6 transgenic mice develop epidermal hyperplasia that progresses to malignant tumors in some animals suggesting that E6 plays a role in both early and late stages of carcinogenesis (Table 12.4). E6 and E7 proteins from highly oncogenic HPV types are more potent than the same proteins derived from HPV types that cause only warts and this distinction probably reflects subtle differences in the activity of the proteins of different HPV types. For example, E6 from benign and oncogenic types of HPV binds to the amino-terminus of p53 but only E6 from the oncogenic types also binds to the cellular E6-AP protein. E6-AP in complex with E6 functions as a ubiquitin ligase for p53 to promote its ubiquitination and degradation.

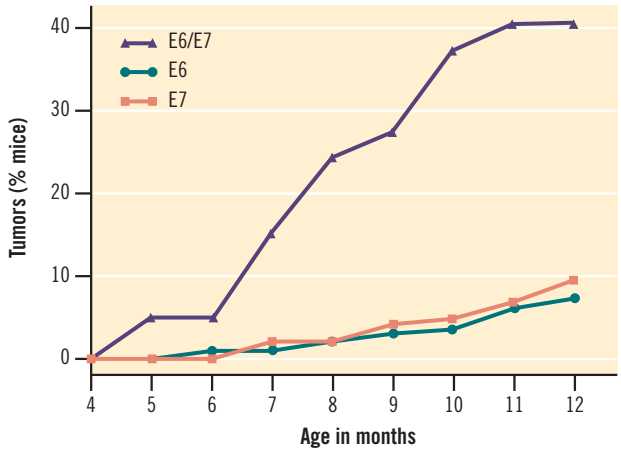

FIGURE 12.4 The synergistic effect of E6 and E7, two oncogenes of HPV. In this experiment, three groups of mice were followed for the frequency of skin tumors: mice transgenic for the E6 gene, for the E7 gene and for both genes. The expression of the oncogenes is controlled by the keratin 14 promoter which is active in the basal cells and hair follicles where stem cells reside. Normal (non-transgenic) mice would show a very low incidence of skin tumors (not shown). After Song S, Liem A, Miller JA, Lambert PF. Human papillomavirus type 16 E6 and E7 contribute differently to carcinogenesis. *Virology* 2000, 267: 141–150, with permission.

Immunological factors

The immune response to HPV antigens plays a major role in the natural history of the benign and malignant lesions caused by these viruses. Observations in humans, although they are anecdotal, are consistent with the ability of the immune response to control HPV-induced lesions:

1. Cutaneous warts are a disease of early childhood and they disappear in most subjects suggesting the development of protective immunity. In adults, benign warts frequently regress 'spontaneously' in normal patients.

Transgenes (number of mice)	Evidence of epidermal hyperplasia		Skin tumors (%)
	Cataracts (%)	Thickened ears or snout (%)	
K14E7 (18)	89	89	0
K14E6 (253)	100	100	13 (total) 9 (malignant)

TABLE 12.4 Skin lesions in transgenic mice bearing the E6 or E7 oncogenes (high copy number) of HPV 16 under control of the human keratin type 14 promoter
After Herber R, Liem A, Pitot H, Lambert PF. Squamous epithelial hyperplasia and carcinoma in mice transgenic for the human papillomavirus type 16 E7 oncogene. *Journal of Virology* 1996, 70: 1873–1881; Song S, Pitot HC, Lambert PF. The human papilloma virus type 16 E6 gene alone is sufficient to induce carcinomas in transgenic animals. *Journal of Virology* 1999, 73: 5887–5893, with permission.

Likewise, genital wart disease is commonly seen in the years after commencement of sexual activity and then usually regresses.

2. Patients who are immunosuppressed due to AIDS, treatment with immunosuppressive regimens, or anti-cancer chemotherapy, are at increased risk of HPV-associated lesions, including cervical carcinoma and lesions are larger, more aggressive and less likely to regress spontaneously than in immunologically unimpaired subjects. This increase of HPV lesions in immunosuppressed patients is most likely due to reactivation of latent infections, rather than acquisition of new infections. When immunosuppression is discontinued, HPV lesions often regress.
3. Patients with dys- or agammaglobulinemia do not experience excessive HPV-associated lesions, suggesting that cellular immunity plays a more important role in the control of HPV disease.

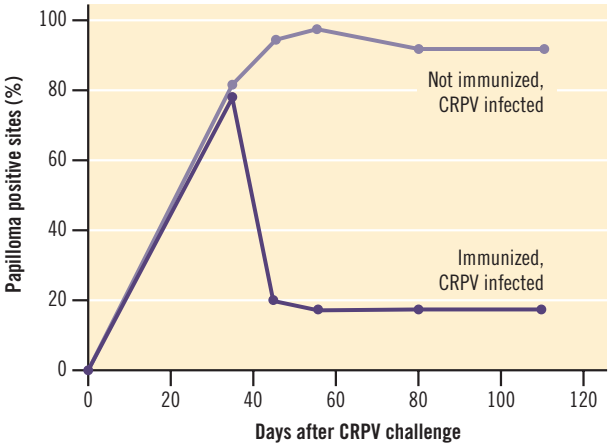

FIGURE 12.5 Immune control of papillomavirus-induced papillomas demonstrated with the best animal model for HPV-induced papillomas, infection of rabbits with the CRPV (cottontail rabbit papillomavirus). In this experiment, rabbits were immunized with E1 and E2 proteins and two weeks later were challenged with CRPV applied to lightly scarified skin. The graph shows the subsequent growth of papillomas in control and immunized animals. After Selvakumar R, Borenstein LA, Lin Y-L, Ahmed R, Wettstein FO. Immunization with nonstructural proteins E1 and E2 of cottontail rabbit papillomavirus stimulates regression of virus-induced papillomas. *Journal of Virology* 1995, 69: 602–605, with permission.

The cotton rabbit papillomavirus (CRPV) is the best animal model for HPV disease and has been used to study the role of immunity in prevention and regression of papillomavirus lesions. A number of studies indicate that immunization of rabbits will provide partial or complete protection against subsequent intracutaneous challenge with CRPV (Figure 12.5). In immunized rabbits, papillomas either fail to develop, are smaller, or regress more frequently than in non-immunized control animals. Both early (E) and late (L) proteins can provide protection, but it is likely that the most important antigens are E1, E2, E6 and E7, since only the early proteins are expressed in the basal stem cell and lower spinous layers.

Polyomaviruses: SV40, a model tumor virus

Polyomaviruses are small double-stranded DNA viruses with a circular genome that is enclosed in a protein capsid. The oncogenic properties of polyomaviruses have been elucidated in studies of two viruses, simian virus 40 (SV40) and mouse polyomavirus (MPV) (Table 12.5). Rhesus macaques are natural hosts of SV40 and wild mice are the natural hosts of MPV.

Polyomaviruses have a small number of genes, that are expressed either early or late during cellular infection. The early genes of SV40 encode two tumor (large T, small t) antigens. Large T is required for viral replication and for transformation. Small t antigen is not required for viral replication, but may be necessary for transformation under some conditions. Transfection of individual SV40 genes shows that large T antigen alone can transform cells (see Table 12.2); transformation is mediated by complexing with pRb and p53 (see Figures 12.2 and 12.3), as well as by other activities. The SV40 late genes encode structural proteins of the virion but are not involved in transformation.

When cells are infected with SV40, there are several possible outcomes:

1. If cells are permissive (for instance, many primate cell types), all the viral genes are expressed, new virus is produced and the cells usually undergo cytolysis with release of virions.

	SV40 virus	Mouse polyomavirus
Natural host	Monkey (Asiatic macaques)	Mouse
Tumors in natural host	Rare	Rare salivary gland tumors
Tumors in experimental hosts	Tumors in newborn hamsters	Tumors in newborn mice
Infection of permissive cultured cells	Productive lytic infection of monkey cells	Productive lytic infection of mouse cells
Infection of non-permissive cultured cells	Transient or permanent transformation	Transient or permanent transformation

TABLE 12.5 Tumorigenic properties of SV40 and mouse polyomaviruses

2. If cells are not permissive (for instance, established mouse cell lines), only the early genes are expressed and the cells are transformed. Under these circumstances, the SV40 genome is usually not integrated and is degraded in a few days, in which case the cells lose their transformed phenotype (transient transformation), since large T antigen must be constantly synthesized to maintain the transformed state. Rarely, the SV40 genome integrates, large T antigen is continuously expressed and the cells are stably transformed.
3. If primary mouse cells are the target, then SV40 can immortalize them to produce a cell line; again, this requires the large T antigen.

When SV40 is used to infect newborn hamsters, a wide variety of tumors is produced. A similar outcome is seen in mice that are transgenic for the SV40 large T antigen. The cellular nature of the tumor is determined by the cell type that is transformed. Infection of newborn animals likely results in tolerance of tumor-specific neoantigens, while adult animals will reject SV40-transformed cells.

It is currently debated whether or not SV40 is maintained in the human population (in addition to selected species of monkeys). SV40 sequences have been detected in certain human tumors, but it is disputed whether this finding represents a polymerase chain reaction (PCR) artefact or implies a causal role for SV40. There are two ubiquitous human polyomaviruses, JC and BK, but neither has been associated with neoplasms. JC virus produces latent infections of the central nervous system and, particularly in immunosuppressed humans, can be activated to cause progressive multifocal leukoencephalopathy. BK virus causes persistent infections of the urogenital tract and is associated with renal disease in immunosuppressed patients.

Adenovirus: oncogenesis in an experimental system

Adenoviruses are ubiquitous human viruses, the majority of which infect the respiratory tract and cause respiratory illness. Infection is self-limiting due to immune surveillance. Although they have not been associated with tumors or cancer in their human hosts, adenoviruses can produce tumors in experimentally infected rodents. In nature, various types of adenoviruses infect epithelial tissues, including respiratory epithelium, conjunctiva and intestinal epithelium. As with HPVs, adenoviruses also require the S phase cellular milieu to complete their replication and they encode proteins that promote S phase re-entry in differentiated cells. In permissive human cells, virus is subsequently produced and released.

Human adenovirus can also infect cultured rodent cells. These cells are not permissive and therefore do not undergo lytic productive infection, but they can be transformed. The transformed cells contain integrated viral genomes, express some of the early proteins (encoded by the E1A and E1B region), exhibit the hallmarks of tumor cells and are no longer subject to the growth restrictions of normal cells.

Although all human adenoviruses transform cultured rodent cells, only some of them are tumorigenic in rats and hamsters. Adenovirus from group A (adenovirus 12, 18) have high oncogenic potential, those from group B (adenovirus 3, 7) are typically oncogenic and the group D adenovirus 9 has lower oncogenicity compared to viruses from groups A and B. Group C and E adenoviruses have not shown oncogenic properties in these animal models.

In vitro cell culture systems and in vivo rodent models have been exploited to analyze adenovirus transformation. Cellular transformation by adenoviruses is accomplished by two regions of the genome, E1A and E1B, both of which encode proteins that are expressed early after infection. The ability to cause tumors in animals also requires another action of adenoviruses, the ability to downregulate the expression of MHC class I proteins on the cell surface, which is also a function of E1A (described in Chapter 4).

E1A and E1B

The E1A proteins have several domains that bind different cellular proteins. The CR1 (conserved region 1) and CR2 domains bind the retinoblastoma protein (pRb) and prevent its action. As discussed already, pRb controls the transition from G1 to S phase (see Figure 12.1) and removing its braking action activates E2Fs and the cell cycle. In addition, the CR1 domain of E1A binds a group of proteins, such as p300, which also act as a regulator of the cell cycle. p300 and related proteins cooperate with p53 to induce transcription of p21, the CDK inhibitor that blocks the cell cycle, and the sequestration of p300 removes another constraint on the cycle (Figure 12.6).

E1B synergizes the action of E1A (Figure 12.7). When E1A alone is introduced into cells, the cells are immortalized at low frequency, if at all. This is because

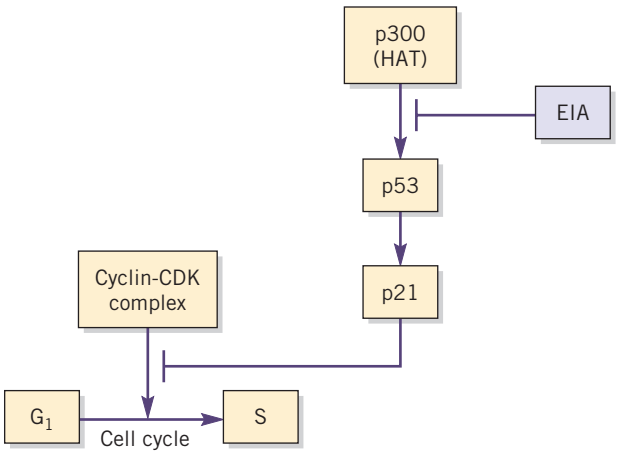

FIGURE 12.6 Adenovirus E1A acts as an oncoprotein by interfering with several brakes on the cell cycle – one of which is illustrated in this cartoon. p300, a HAT (histone acetyltransferase) protein promotes the transcription of p53 by altering chromatin from a condensed to an open form. p53, in turn, acts as a transcription factor for p21, a protein that binds and sequesters cyclin-CDK (cyclin-dependent kinases) complexes. Cyclin-CDK complexes activate the cell cycle and p21 acts as a brake on the cycle. E1A sequesters p300, retards the downstream activation of p21 and removes the p21 brake on the cell cycle.

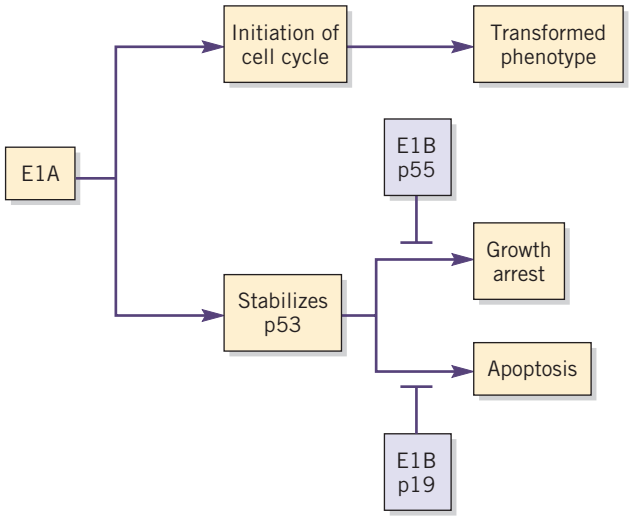

FIGURE 12.7 The oncogenic activity of adenovirus is associated with the E1A and E1B proteins, both of which are encoded by early (E) genes. These proteins work in a complementary manner to transform cells. E1A drives resting cells into cell cycle and thence into a transformed phenotype (see Figure 12.6). E1A also stabilizes p53, an anti-oncoprotein and p53 initiates growth arrest and apoptosis. Thus cells transformed with E1A alone undergo transient transformation, but the transformed foci quickly die. Adenovirus E1B encodes a p55 and a p19 protein; p55 blocks growth arrest and p19 blocks the apoptosis pathway. Thus, cells co-transformed with both E1A and E1B genes undergo stable transformation.

the expression of E1A alone not only triggers S phase entry, but also elevates p53 levels which induces apoptosis. E1B encodes two proteins, p55 and p19, which block the action of p53 in different ways. p55 binds p53 and blocks the upregulation of p21 (see Figure 12.2). The apoptosis action of p53 is mediated by the induction of 'cell death' proteins, such as Bax, that trigger the caspase pathway leading to apoptosis; p19 binds and sequesters Bax, thereby preventing apoptosis.

In mice injected with human adenovirus, many cell types and tissues are infected, including liver, muscles and hematopoietic cells. Defective adenovirus (missing E1A and/or E1B) has been used as a vehicle for human gene transfer. Its wide range of tissue tropism is an advantage because many cell types or tissues can be transduced, but its potential widespread cytotoxicity is also a limitation. In addition, because adenovirus engineered to delete E1B promotes apopotosis, it is being tested as an oncolytic agent via intratumoral injection of solid tumors, including some head and neck cancers.

Herpesviruses: Epstein-Barr virus (EBV)

EBV is a member of the γ subfamily of herpesviruses, characterized by their ability to infect lymphoid cells. Biologically, EBV differs widely from adenoviruses in its oncogenic activity, due in large part to its ability to transform and immortalize human primary B cells. EBV is associated with a range of lymphoid and epithelial cancers in humans. Typically, EBV is a less potent carcinogen than the small oncogenic DNA viruses.

The majority of the adult human population is persistently infected with EBV. Initial infections occurring in childhood are usually asymptomatic, while an estimated third of infections in adolescents or young adults are associated with infectious mononucleosis (a transient self-limited systemic illness with sore throat, malaise and fever). EBV is associated with one of several neoplasms, Burkitt's lymphoma, nasopharyngeal carcinoma or Hodgkin's disease, that usually occur long after initial infection. EBV acts as a co-carcinogen in a multistep process leading to the development of a number of cancers. However, EBV is known to be directly capable of driving some cancers, including post-transplant lymphoproliferative disease (PTLD) in transplant patients and AIDS-associated lymphomas; both are typically associated with the immunocompromised state. Figure 12.8 summarizes the likely pathogenesis of EBV-associated infections.

B lymphocytes

EBV targets B lymphocytes via its major glycoprotein, gp350, that serves as the virus attachment protein. gp350 binds to its B cell receptor, CR2 (complement receptor type 2, also called CD21), that is expressed mainly on B cells. However, entry is a complex process and a subsequent step involves a tri-molecular complex of viral glycoproteins, gp42, gH and gL, which binds to a co-receptor, class II MHC, via gp42.

Upon entry into B lymphocytes, there is partial expression of the viral genome, persistence in a latent state and immortalization of the infected cell. Peripheral blood lymphocytes obtained from seropositive donors, depleted of T cells and cultured appropriately, will usually yield immortalized lymphoblastoid cell lines. These immortalized cell lines are typically generated during the process of ex vivo culture. Cell culture can trigger the lytic cycle in some persistently infected B cells and the virus released from these cells recruits uninfected B cells leading to the development of lymphoblastoid cell lines (see Figure 12.8).

Infected B cells account for the persistence of the virus in humans, but rarely release virus and are probably not responsible for viral shedding. In infected humans, therapeutic whole body ablation of the lymphoid population (which leaves the epithelial cells intact) eradicates EBV infection. This suggests that the virus depends for its maintenance mainly upon long-lived transformed B cells which are present at a frequency of 10^{-5} to 10^{-6} among peripheral blood B cells.

Epithelial cells

In addition to B cells, EBV infects epithelial cells in the oral cavity. EBV enters epithelial cells (that lack CR2 and HLA class 2 molecules) via a different entry pathway than it uses for B cells. A bimolecular complex of viral glycoproteins, gH and gL, are required. Within the epithelium, EBV is only partially expressed in the basal cells but completes its replicative cycle in the more superficial differentiated cells in the upper spinous and granular layers, in a manner reminiscent of HPV (see Figure 12.3). Productive infection of epithelial cells is probably responsible for shedding of infectious EBV, which can be isolated

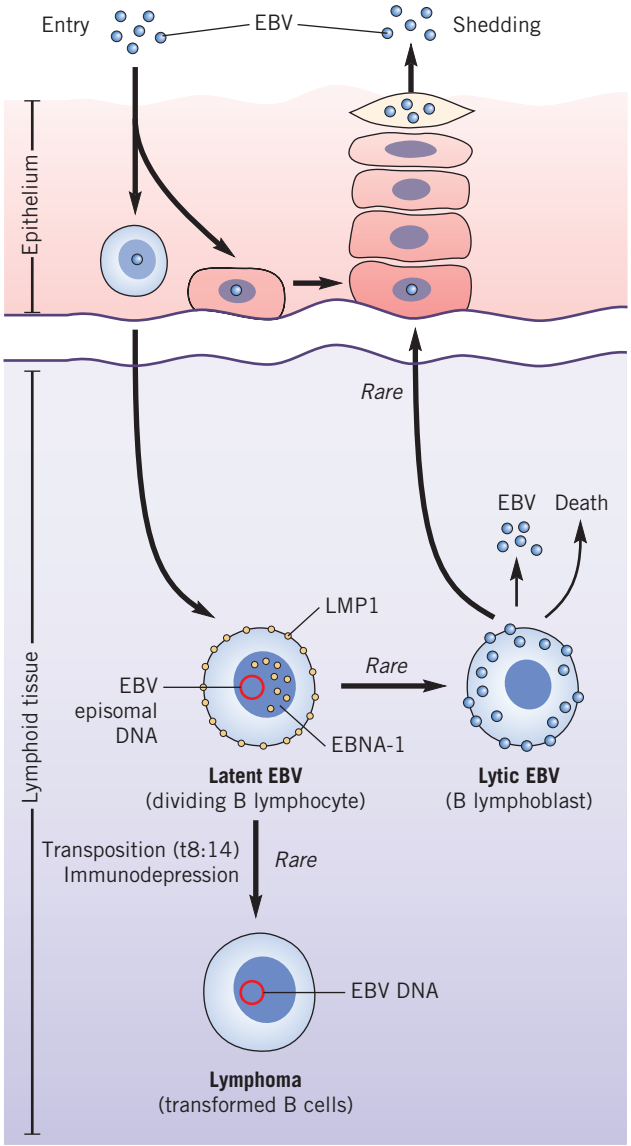

FIGURE 12.8 Diagrammatic summary of possible pathogenesis of EBV and Burkitt's lymphoma. The virus initially infects epithelial cells and B lymphocytes. In epithelial cells, EBV causes both latent and lytic infection, the latter resulting in virus shedding into the oral cavity. EBV persists in resting B cells as a latent infection which is rarely activated to produce new virus. During latent persistence in resting memory lymphocytes, no EBV antigens are expressed but two segments of the genome are transcribed. When EBV infects dividing lymphocytes in the periphery or in lymph node germinal centers, latent antigens are expressed and these cells can undergo transformation into tumor cells. Tumors included a variety of lymphomas, such as Burkitt's lymphoma, which is frequently associated with a chromosomal transposition (t8:14) that upregulates the *c-myc* oncogene. EBNA: Epstein-Barr nuclear antigen; LMP: latent membrane protein.

from throat swabs in many seropositive carriers. Likely this accounts for spread of the virus from host to host, but is not a pathway to oncogenesis (see Figure 12.8).

EBV has evolved a novel strategy that permits it to shuttle between B cells and epithelial cells. Virus released from B cells has a bimolecular glycoprotein complex that facilitates entry into epithelial cells and virus released from epithelial cells expresses mainly the trimolecular complex that facilitates entry into B cells.

Latent infection and cellular immortalization

Following viral entry into human B lymphocytes, the nucleocapsid is released into the cytosol and the linear double-stranded DNA genome is released. The genome, with unspecified viral proteins, passes through a pore in the nuclear membrane. Once in the nucleus, the genome remains in a linear form until the host cell has passed through the next cell division, when the genome is circularized. During latency, the genome is maintained as a circularized replicating episome, which replicates before or during cell division, so that there may be several copies per infected cell.

During initial infection of cultured B lymphocytes, a number of EBV genes are expressed and several of the expressed proteins are required for immortalization (Table 12.6). However, cultured EBV-positive B cell clones from lymphomas express mainly two EBV proteins, EBNA-1 and LMP1. EBNA-1 is a nuclear protein required to maintain and replicate the viral genome as an unintegrated episome. LMP1, an integral plasma membrane protein, has a pleiotropic action including constitutive activation of some B cell growth factor receptors, such as TRAF (TNF receptor associated factor) 1 and 3, which regulate B cell proliferation (Figure 12.9). LMP1 has been considered an oncogene, since transgenic mice expressing this protein develop B cell lymphomas. Importantly, EBV-derived B cell clones generated in vitro from primary B cells or from PTLDs or AIDS lymphomas express all the major latent transcripts.

EBV-associated tumors cannot be attributed to the action of LMP1 alone and transformation involves a multistep process. Immortalization of resting B cells is a first step in tumorigenesis and can be accomplished by EBNA-1 and LMP1 alone. Progression to the transformed phenotype is a second step that requires a number of cellular events, such as upregulation of telomerase (to counter shortening of chromosomes), downregulation or mutation of anti-oncogenes such as pRb and p53, and blockade of apoptosis pathways.

Immune control of EBV

As mentioned above, a high proportion of normal subjects are carriers of EBV and lymphoblastoid cells lines can be isolated from their peripheral blood in many instances. When human EBV carriers are immunosuppressed, as occurs in transplantation recipients or in AIDS, they are subject to lymphoproliferative disorders such as immunoblastic (Hodgkins and non-Hodgkins) lymphomas and the proliferating cells are often EBNA (EBV nuclear antigen) positive. It is assumed that these disorders represent EBV-transformed B cells that have escaped immune surveillance. This implies that in normal EBV carriers, cellular immune responses control the proliferation of EBV-immortalized lymphoblastoid clones.

Burkitt's lymphoma

First described by Dennis Burkitt as a fatal lymphoma of the head and neck in African children, three categories of Burkitt's lymphoma are now recognized. The originally

EBV gene	Localization	Function (all are essential for immortalization)
EBNA-1	Nuclear	Required for the maintenance of the EBV episome
EBNA-2	Nuclear	Transactivator of viral and cellular genes
EBNA-3A, 3C	Nuclear	Transcription factor, regulator of viral and cellular genes
LMP1	Plasma membrane	Main transforming protein of EBV Pleiotropic functions, including constitutive activation of cellular receptors TRAF 1, TRAF 3 Ligand-independent activation of several signaling pathways

TABLE 12.6 EBV (Epstein-Barr virus) immortalizes B lymphocytes by establishing a persistent latent infection. During latent infection a number of EBV genes are expressed. This table lists some of the latency-associated genes that are essential for immortalization, based on experiments with EBV mutant viruses
EBNA: Epstein-Barr nuclear antigen; LMP: latent membrane protein; TRAF: TNF receptor-associated factor. After Damania B. Oncogenic γ-herpesviruses: comparison of viral proteins involved in tumorigenesis. *Nature Reviews Microbiology* 2004, 2: 656–668, with permission.

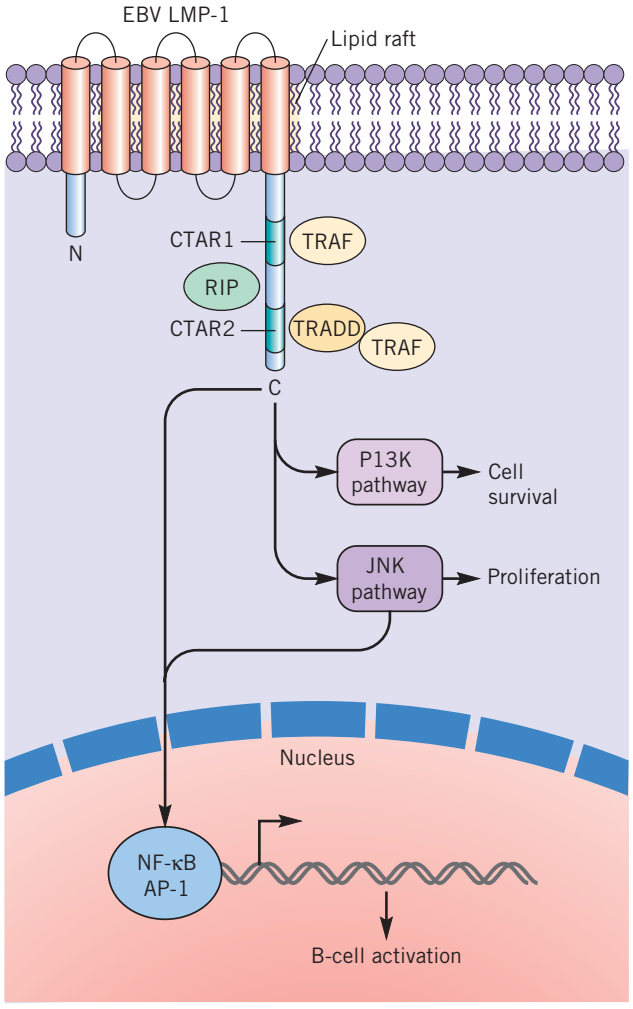

FIGURE 12.9 Transforming pathways of the LMP1 protein of EBV. EBV LMP1 can interact with multiple TRAFs and TRADDs leading to B cell activation through the induction of cellular transcription factors AP-1 and NF-κB. AP-1: activating protein 1; CTAR: C-terminal activator regions 1 and 2; EBV: Epstein-Barr virus; JNK: Jun N-terminal kinase; LMP: latent membrane protein; NFAT: nuclear factor of activated T cells; NF-κB: nuclear factor κB; PI3K: phosphatidylinositol 3-kinase; RIP: receptor-interacting protein; TRADD: tumor necrosis factor receptor-associated death domain; TRAF: tumor necrosis factor receptor-associated factor. After Damania B. Oncogenic γ-herpesviruses: comparison of viral proteins involved in tumorigenesis. *Nature Reviews Microbiology* 2004, 2: 656–668, with permission.

described endemic form is particularly frequent in West Africa and coastal New Guinea, there is a worldwide rarer 'sporadic' form and a third form seen as a complication of AIDS. One salient feature of Burkitt's lymphoma is that EBV DNA can be detected in tumor cells in >95% of endemic cases and in less than half of the sporadic and AIDS-associated cases. In individual patients, the EBV DNA sequences are uniform, suggesting that each tumor represents the clonal expansion of a single transformed cell.

The second characteristic feature of Burkitt's lymphoma is that the lymphoma cells exhibit a chromosomal translocation (t8:14) between chromosome 8 and (most frequently) chromosome 14, resulting in the relocation of *c-myc*, a proto-oncogene on chromosome 8, downstream from the enhancer regions for immunoglobulin heavy or light chains. Also, mutations in *c-myc* are frequent. In proliferating B lymphoblastoid cells, these genetic alterations lead to constitutive over-expression of *c-myc*, which acts as a transcription factor important in the cell cycle, driving B lymphocytes to unrestrained proliferation.

A likely third factor in the multistep process leading to cancer formation, is loss of immune surveillance, which could increase the proliferation of lymphoblastoid cell lines both before or after chromosomal translocation. Endemic Burkitt's lymphoma is co-extensive with *Plasmodium falciparum* malaria and it is postulated that chronic malaria would reduce immune competence; consistent with this hypothesis is the increased risk of Burkitt's lymphoma in patients with AIDS.

In summary, EBV immortalizes B lymphocytes through the action of several of its gene products that convert infected cells to a constitutive state of proliferation. EBV infection may initiate Burkitt's lymphoma, but only as part of a multistep process (see Figure 12.8) that includes chromosomal translocation of the *c-myc* proto-oncogene and immunosuppression.

Herpesviruses: HHV8 and Kaposi's sarcoma

Kaposi's sarcoma (KS) is a neoplasm of the skin and viscera that is associated with HHV8 (human herpesvirus 8, also called Kaposi's sarcoma-associated herpesvirus).

Epidemiological and pathological evidence strongly suggests that HHV8 plays a causal role in KS, but the multiple mechanisms are only partially understood. The discovery of HHV8 is an instructive example of the use of molecular methods to unravel the etiology of an infectious disease (see Chapter 15) and illustrates the complexities of viral oncogenesis.

History and background

KS was described over 100 years ago as a relatively uncommon sarcoma of the skin in older men in Eastern Europe and the Mediterranean region. In the 1960s, prior to the emergence of AIDS, a more aggressive form of KS was reported in East African children. In the 1980s, KS achieved new prominence as one of the diseases associated with AIDS. In the USA, KS is mainly seen in gay men with AIDS and over 95% of cases in the USA are in this group. The incidence of KS in US gay men is over 10 000-fold that in the general population and more than 10-fold greater than in other AIDS patients, such as injecting drug users and blood recipients. These observations led to a number of hypotheses regarding the etiology of KS (see below).

The histopathology of KS is unusual for sarcomas, since the lesions consist of many different cell types, of which the dominant one is the spindle cell, thought to represent a transformed endothelial cell. In addition, tumors are infiltrated with inflammatory cells and exhibit large numbers of newly formed vascular elements. Cultured spindle cell lines secrete pro-inflammatory and angiogenic factors, suggesting that the characteristic spindle cell is responsible for the cellular composition of KS tumors.

Etiology

To explain the epidemiological observations, one hypothesis suggested that KS was caused by a previously undetected infectious agent. Searching for footprints of such a putative agent, Chang and colleagues discovered a previously undescribed herpesvirus, since named HHV8 (see Chapter 15). The discovery of HHV8 as a putative causal agent of KS led to serological surveys that partially explained its enigmatic epidemiological patterns (Table 12.7). The great majority of patients with KS are infected with HHV8, consistent with the hypothesis that HHV8 plays a critical role in the etiology of KS. The prevalence of HHV8 is much more frequent in gay men than in drug injectors or recipients of blood or blood products, explaining why KS is much more frequent in homosexual men than in other AIDS risk groups. The data imply that HHV8 is a sexually transmitted disease but is not transmitted by blood, in marked contrast to HIV; this difference has yet to be explained. The much higher prevalence of KS in HIV-positive gay men with AIDS than in HIV-positive gay men without AIDS is consistent with a role of immunosuppression as a co-factor in the development of KS. This is supported by the observation that effective treatment of AIDS with HAART (highly active antiretroviral therapy) leads to arrest or remission of KS lesions. Finally, prospective studies of gay men showed that subjects who were infected with both HIV and HHV8 (in either order) developed KS at a high incidence, while those infected with only HHV8 only did not develop KS (Figure 12.10).

Pathogenesis of KS

The role of HHV8 in the pathogenesis of KS is not completely understood, but a general picture has emerged. HHV8 is a γ-herpesvirus that bears considerable similarity to EBV. In many HHV8-seropositive subjects, HHV8 can be detected in peripheral blood B lymphocytes, which maintain the virus as a persistent infection. Furthermore, HHV8 is associated with a variety of unusual lymphoproliferative neoplasms, such as primary effusion lymphoma,

Subjects	Frequency of HHV8 antibody	
	HIV negative	HIV positive
Kaposi's sarcoma		
Endemic KS (Greece)	17/18 (94%)	
AIDS cases		84/103 (82%)
Without Kaposi's sarcoma		
Homosexual men	8/65 (12%)	10/33 (30%)
Injecting drug users	0/25 (0%)	0/38(0%)
Hemophiliacs		0/26 (0%)

TABLE 12.7 Prevalence of HHV8 infection and Kaposi's sarcoma (KS) shows that most patients with KS are infected with HHV8, consistent with the hypothesis that HHV8 plays an essential role in the etiology of KS. Regardless of HIV status, HHV8 is much more prevalent in homosexual men than other HIV risk groups such as injecting drug users and patients with hemophilia. This could explain why AIDS-associated KS is mainly seen in homosexual men compared to AIDS patients from other risk groups. The serological test for HHV8 infection used in this study was probably suboptimal for sensitivity
Modified from Simpson GR, Schulz TF, Whitby D *et al.* Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibody to recombinant capsid protein and latent immunofluorescence antigen. *Lancet* 1996, 348: 1133–1138, with permission.

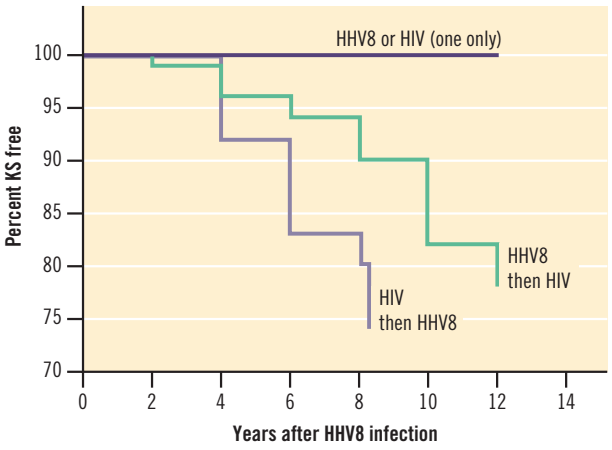

FIGURE 12.10 The cumulative incidence of Kaposi's sarcoma (KS) in a group of gay men infected with HHV8 and with HIV. Infection with HIV followed by HHV8 infection carried a higher risk than HHV8 infection followed by HIV infection. Subjects infected with HHV8 only or with HIV only did not develop KS. After Jacobson SP, Jenkins FJ, Springer G *et al.* Interaction of human immunodeficiency virus type 1 and human herpesvirus type 8 infections on the incidence of Kaposi's sarcoma. *Journal of Infectious Diseases* 2000, 181: 1940–1949, with permission.

suggesting that it may transform B cells. Some cell lines derived from these unusual lymphomas carry HHV8 as a persistent infection in which the viral DNA exists as an episome. Most of the cells in such cultures are latently infected and express a limited number of HHV8 transcripts, while productive replication with expression of the whole genome and generation of infectious virus is seen in only a few cells. The relationship between lytic and latent phases of HHV8 infection is a subtle one, that is controlled by the ability of the major latency associated antigen (LANA) to repress another viral protein, the RTA (replication and transcription activator) by blocking its downstream signaling pathway.

Many spindle cells in the KS lesion are positive for HHV8 DNA and most of these express transcripts typical of latent infection, while a few express whole genome transcripts. HHV8 encodes a number of genes that could play a role in cell transformation or tumorigenesis. These include proteins that resemble cyclins, chemokines, cytokines or G protein-coupled receptors, as well as proteins similar to bcl-2 which can block apoptosis. In addition, the major latency associated antigen, LANA-1, modulates the transcription of pRb and p53. It appears likely that these and other HHV8-encoded proteins play a role in the cellular proliferation of endothelial and B cells that is characteristic of HHV8-associated neoplasms. An in vitro model has been developed, using a line of immortalized human dermal microvascular cells which, when exposed to HHV8, become latently infected and acquire a spindle-shaped morphology. These cells exhibit loss of contact inhibition and anchorage-independent growth and this model reproduces many of the characteristics of KS in humans, which strongly supports the etiological role of HHV8 in this disease.

One example of multiple mechanisms whereby HHV8 immortalizes or transforms infected cells is its ability to activate the transcription of genes, such as *myc*, that drive the cell cycle (Figure 12.11). The LANA (latency-associated nuclear antigen) protein has been shown to bind a cellular cytoplasmic protein, GSK-3β and sequester it in the nucleus. The normal role of GSK-3β is to cooperate with other cytoplasmic proteins to act as a brake on the Wnt signaling pathway; the Wnt pathway frees β-catenin to move to the nucleus where it acts as a transcription factor, driving the cell cycle. When LANA binds GSK-3β, it removes this normal brake on Wnt signaling, leading to unregulated cell division.

In summary, Kaposi's sarcoma illustrates the multifactorial nature of virus-induced neoplasia. In this instance, it appears that HHV8, a newly described γ-herpesvirus, is the etiological agent that infects and transforms endothelial cells converting them into the spindle cells characteristic of KS lesions. Transformation is probably mediated by multiple viral genes that encode proteins that upregulate the cell cycle, constitutively activate growth factor pathways and block apoptosis. The transformed cells have limited tumorigenic capacity and are normally controlled by immune surveillance. However, immunosuppression, associated with AIDS, holoendemic malaria, or aging, markedly increases the risk of overt KS disease.

REPRISE

The oncogenic DNA viruses transform cells by mechanisms that differ from those used by the retroviruses. The small oncogenic DNA viruses (adenoviruses, polyomaviruses, papillomaviruses) have transforming genes that inactivate anti-oncogenes, such as p53 and pRb, which normally act as brakes on the cell cycle. In many instances, the large oncogenic DNA viruses (some herpesviruses and poxviruses) immortalize cells by interacting with extracellular growth factors and the intracellular signaling systems that respond to growth factors, in addition to inactivating tumor suppressors. In addition, they may may encode homologs of cellular proteins that

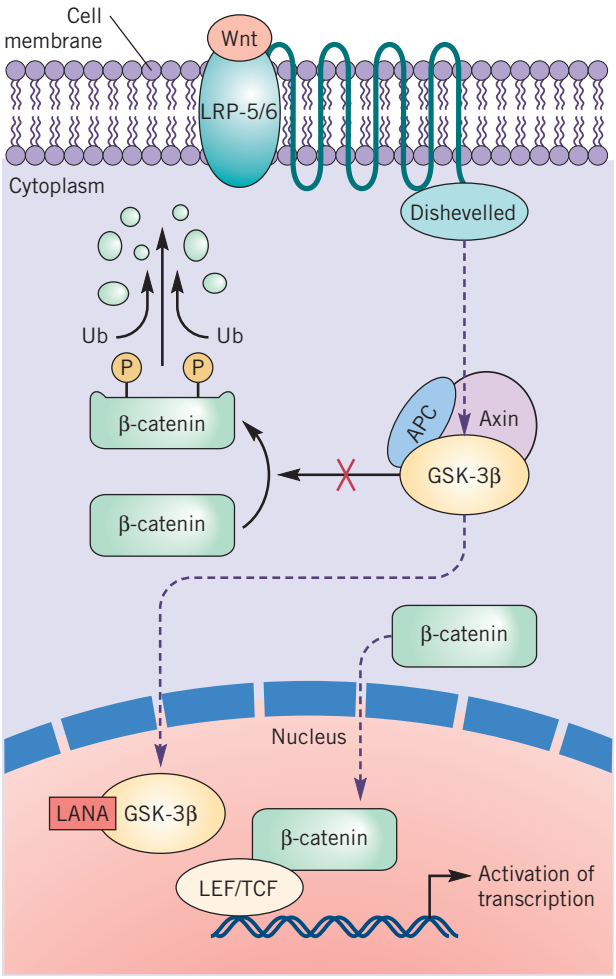

FIGURE 12.11 HHV8 stimulates cell division by many mechanisms, one of which is illustrated here. Wnt proteins are cytokines that drive cells into cycle by binding to receptors such as LRP-5/6, which signals via the Dishevelled protein to initiate a complex with GSK-3β. GSK-3β normally acts as a brake on the cell cycle by phosphorylating β-catenin and sending it to the ubiquitin-proteosome pathway. Wnt signaling binds GSK-3β, removing this brake. In a similar fashion, LANA (latency associated nuclear antigen), an HHV8 protein, binds and sequesters GSK-3β, removing the brake and permitting β-catenin to enter the nucleus and (in concert with other transcription factors) to start the transcription of genes such as MYC that activate the cell cycle. GSK: glycogen synthase kinase; LEF: lymphoid enhancing factor; TCF: T cell factor; LRP-5: LDL receptor-related protein; APC: adenomatosis polyposis coli; MYC: myxomatosis; Ub: ubiquitin. After Boshoff C. Kaposi virus scores cancer coup. *Nature Medicine* 2003, 9: 261–262, with permission.

activate the cell cycle or interfere with intracellular signaling that leads to apoptosis. The oncogenic DNA viruses reinforce some general themes regarding virus-induced tumorigenesis. Cancer is a multistep process and additional mutations in host genes are usually necessary for carcinogenesis initiated by oncogenic DNA viruses. Also, in many instances, the appearance and aggressiveness of tumors is modulated by immune responses against both viral gene products and tumor antigens, indicating the potential for preventive and therapeutic vaccines.

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Chapter 13

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Host Susceptibility to Viral Diseases

Neal Nathanson and Margo A. Brinton

INTRODUCTION

A central theme of this book is that the outcome of a viral infection depends upon both the parasite and the host. Just as a particular virus can vary in its virulence, so members of a given host species can vary in their response to a single virus. This diversity is apparent during an outbreak of smallpox or poliomyelitis, when individual infections range from inapparent to fatal. However, potential differences in the route of infection and in the strain and dose of virus could account for the observed variance in illness and mortality in natural infections, so that the role of host determinants is ambiguous. By contrast, experimental comparison of the responses of inbred strains of animals to a standardized virus inoculum provides convincing evidence of host variation in the outcome of infection.

This chapter describes host genes that have a striking influence upon the course of particular viral infections. Most experimental studies employ mice because of the availability of a vast number of inbred strains, permitting comparisons between animals with different genetic backgrounds. It might be predicted that immune response diversity could explain genetic variation in susceptibility but, in most mouse models, non-immune determinants are operative.

Similar genetic determinants undoubtedly exist in other species although the data are sparse. For instance, domestic rabbits are considerably more susceptible to the oncogenic effects of cotton rabbit papillomavirus (CRPV) than are cottontail rabbits, but there are no tools available to map the responsible genetic loci. By implication, genetic determinants also operate in humans, although it has been difficult to test this hypothesis. However, recent studies of HIV/AIDS and other human viruses have revealed a number of such genetic loci, which undoubtedly represent only the tip of the iceberg.

In addition to genetic determinants, other host factors, such as age and nutritional status, can play a significant role in the outcome of a viral infection. These variables are described at the end of this chapter.

In nature, many viruses are quite species-specific while others infect a wide range of species. Susceptibility differences between species (in contrast to variation among members of a single species) are discussed in Chapter 15, which describes how viruses cross the species barrier.

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GENETIC DETERMINANTS OF SUSCEPTIBILITY IN MICE

Overview

There are many inbred strains of mice that can be readily tested for their susceptibility or resistance to viruses. If any single virus is compared in enough mouse strains, some variability in disease or mortality will be seen, as illustrated in Table 13.1. From studies of these mouse models, a number of generalizations can be made.

- In many instances, differences in susceptibility are determined by a single genetic locus, as shown by testing F1 and F2 crosses. Either resistance or susceptibility can be dominant.
- Determinants identified in animal experiments may or may not be observable in cultured cells derived from resistant and susceptible hosts.
- Most genetic determinants do not map to the MHC (major histocompatibility complex) locus (H-2 in the mouse) and so are due to non-immune mechanisms. However, a few genetic determinants do map to the MHC locus (H-2 in the mouse).
- Most genetic determinants affect the response to a single virus or virus family. Therefore, each determinant is unique and different resistance loci map to different chromosomes.
- For murine retroviruses, some genetic loci represent endogenous viral sequences that have been incorporated into the host genome (discussed in Chapter 11).

A representative list of genetic loci that determine susceptibility is shown in Table 13.2. A few of these will be described to illustrate their effects upon viral infection and cellular mechanisms.

Non-immunological determinants

The Mx gene, interferon and influenza virus

The Mx gene is a classical example of a genetic determinant of disease susceptibility that has been studied exhaustively, although much remains to be elucidated about the mechanism of its action. It was observed in the 1960s that most strains of inbred mice are susceptible to influenza and related orthomyxoviruses, but the A2G mouse strain is resistant (Figure 13.1), based on the severity of disease. Furthermore, there is a correlation between susceptibility to disease and level of virus replication in the lung. When F1 and F2 hybrids between the two strains of mice are tested, susceptibility segregates as a single genetic locus and resistance is dominant (Table 13.3). The effect of this genetic locus (designated as *Mx* for myxovirus) is limited to influenza virus and other single-stranded negative sense RNA viruses.

Macrophages from susceptible and resistant mice show the expected Mx phenotype when tested soon after culture, but two weeks later the resistant cells have become susceptible. The loss of resistance correlates with a cessation of interferon production by macrophages from A2G resistant mice. The Mx resistant phenotype (resistance to influenza virus) is dependent upon interferon since treatment with anti-interferon antiserum abrogates resistance to influenza virus (Table 13.3). However, the Mx locus does not code for interferon itself, since both susceptible and resistant mice produce similar interferon responses to viral infection.

Interferon α/β produces its effects indirectly by inducing the expression of a plethora of cellular genes (see Figure 5.4) and one of these is the Mx1 gene (Figure 13.2). The Mx1 gene has been cloned and its product in resistant A2G mice is a 72 kD nuclear protein, which functions as a GTPase. Influenza-susceptible mouse strains have either a nonsense mutation or deletion that disrupts the reading frame of this protein and no Mx1 peptide is detected. Mx1 interacts with the viral ribonucleoprotein (vRNP) complex, which could prevent nuclear transport of incoming vRNPs, or could interfere with transcription and replication of vRNPs. The detailed mechanism of Mx1 protein action remains to be fully elucidated.

Viral replication, the Flv gene and flaviviruses

Flaviviruses, named after yellow fever virus, cause encephalitis when injected intracerebrally in mice. Most laboratory strains of mice are susceptible to flavivirus-induced disease, but a few strains, such as the PRI (Princeton Rockefeller Institute) strain are resistant. When crosses were made between susceptible and resistant mice, the trait segregated as a single autosomal gene and resistance was dominant. The locus controlling flavivirus susceptibility was named *Flv* and has been mapped to mouse chromosome 5.

Mouse strain	H-2 haplotype	Female dead/total (% mortality)	Male dead/total (% mortality)
A.WY/SnJ	a	60/62 (97)	50/51 (98)
A.SW/SnJ	s	34/37 (92)	33/35 (94)
C57BL/6J	b	50/66 (73)	16/19 (73)
B10.A	a	22/35 (63)	8/16 (50)
A/J	a	13/22 (59)	Not done
C57BL/10ScN	b	47/93 (50)	35/42 (73)
DBA/2J	d	4/40 (10)	4/9 (44)
BALB/c	d	1/52 (2)	21/50 (42)
CBA/J	k	0/56 (0)	2/34 (6)
SJL/J	s	0/74 (0)	0/62 (0)

TABLE 13.1 Different mouse strains can differ in their susceptibility to a given virus. In this example, mice were inoculated with rabies virus, $10^{7.7}$ mouse ic LD50 by the intraperitoneal route and followed for 21 days to determine mortality. Mouse strains differed markedly in their susceptibility and this did not correlate with the H-2 (MHC) haplotype. Also, there was some suggestion that female mice were less susceptible than were males
After Lodmell DL. Genetic control of resistance to street rabies virus in mice. *Journal of Experimental Medicine* 1983, 157: 451–460, with permission.

Virus (Family)	Disease or effect	Locus designation (number of genes)	Dominant trait	Maps to H-2 (chromosome)
Non-immune				
Influenza (Orthomyxo-viridae)	Pneumonia	*Mx* (1)	Resistance	No (C16)
Flaviviridae	Encephalitis	*Flv* (1)	Resistance	No (C5)
Rabies (Rhabdoviridae)	Encephalitis	(1)	Resistance	No
MHV (Coronaviridae)	Hepatitis (MHV-3)	*Musfbp* (1)	Susceptibility	No
	Death	*Hv-1* (1)	Susceptibility	No
	Infection	*Hv-2* (1)	Susceptibility	No (C7)
MuLV (Retroviridae)	Susceptibility to infection	*Fv-1* (1)	Resistance	No (C4)
Herpes simplex (Herpes)	Encephalitis	(2+)	Resistance	No
Ectromelia (MP)(Pox)	Mousepox	Rmp-1 (1)	Resistance	No
Immune				
LCMV (Arena)	Immune response	(?)	?	Yes (C17)
MuLV (Retro)	Erythroleukemia	Rfv-1 (1)	Co-dominant	Yes (C17)
	Erythroleukemia	Rfv-2 (1)	Co-dominant	Yes (C17)
	Erythroleukemia	Rgv-1 (1)	Co-dominant	Yes (C17)
MPV (Polyoma)	Tumor induction	(1)	Resistance	Yes (C17)

TABLE 13.2 Representative mouse genetic loci that influence the outcome of viral infections
Adapted from Brinton M. Host susceptibility to viral diseases, in Nathanson N, Ahmed R, Gonzalez-Scarano F *et al.* (eds), *Viral pathogenesis*, Lippincott-Raven Publishers, Philadelphia, 1997, with permission.

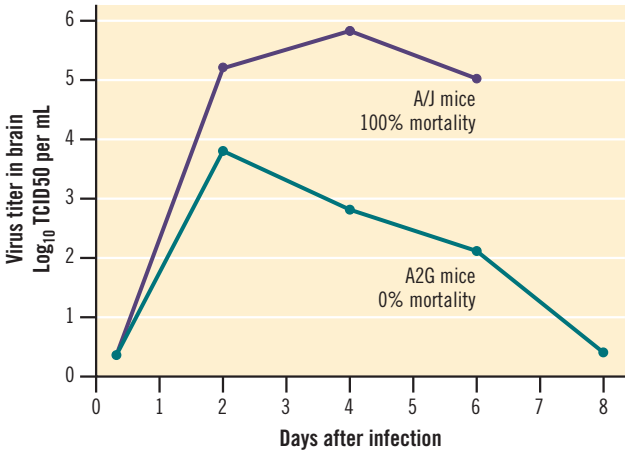

FIGURE 13.1 Inbred strains of mice may exhibit dramatic differences in disease susceptibility to specific viruses. In this example, A/J mice, like most other strains, are susceptible while A2G mice are resistant. Both strains of mice were injected intracerebrally with 70 ic LD50 (as determined in susceptible mice) of a neurotropic strain (WSN) of a type A influenza virus and observed for symptoms and assayed for replication in the brain. The susceptible A/J mice supported a higher level of replication and died, while resistant A2G mice survived. After Fiske RA, Klein PA. Effect of immunosuppression on the genetic resistance of A2G mice to neurovirulent influenza virus. *Infection and Immunity* 1975, 11: 576–586, with permission.

Mouse strain	Genotype	Treatment	Mortality dead/total	Log10 virus titer
A/J	r/r	None	4/4	6.0
A2G	R/R	None	0/4	3.7
F1	R/r	None	0/4	3.5
A/J	r/r	AIF	4/4	6.0
A2G	R/R	AIF	4/4	6.3
F1	R/r	AIF	4/4	6.5

TABLE 13.3 The progeny of genetic crosses between inbred strains of mice can be used to determine the inheritance of susceptibility or resistance. In this example, influenza A virus was used to test A/J mice (susceptible) and A2G mice (resistant) and the results with F1 hybrids (A/J X A2G) showed that resistance was dominant. In addition, treatment of resistant animals with anti-interferon antiserum (AIF) abrogated resistance, indicating that interferon played a role in the resistance phenotype. Virus titers were determined on blood samples obtained two days after infection and are expressed as EID50 (50% egg infectious doses)
After Haller O, Arnheiter H, Gresser I, Lindenmann J. Genetically determined, interferon-dependent resistance to influenza virus in mice. *Journal of Experimental Medicine* 1979, 149: 601–612, with permission.

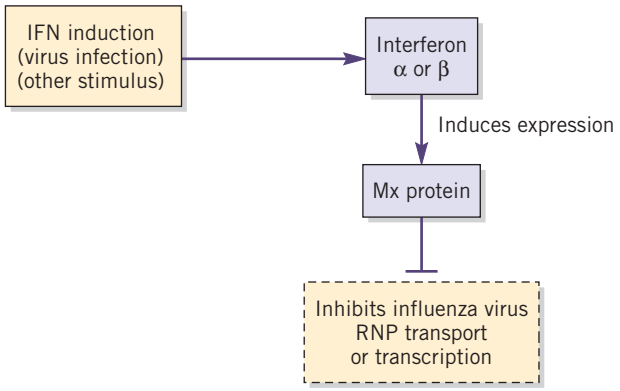

FIGURE 13.2 If a genetic determinant of host susceptibility can be mapped to a single locus, it should encode a specific host protein. One of the best studied examples is the Mx protein, a cellular protein whose normal function is unknown. The *Mx* gene can be induced by interferon and establishes a state of resistance against group A influenza viruses. The Mx protein appears to act by blocking either intracellular transport of influenza virus RNP (ribonucleoprotein) complexes or their transcription.

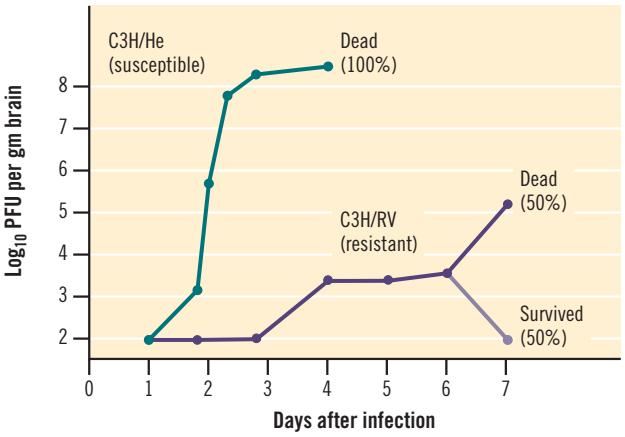

FIGURE 13.3 Effect of a genetic locus (Flv) that influences susceptibility to flavivirus-induced disease. Two strains of mice are compared, C3H/He, an inbred strain that is susceptible, and C3H/RV, a congenic strain that is identical except for ~31 centimorgan region from the resistant PRI strain that contains the resistance allele at the *Flv* locus. Adult mice were injected intracerebrally with $10^{5.5}$ PFU of West Nile virus. All resistant animals exhibited lower virus titers, but some eventually died while others survived. After Brinton M. Host susceptibility to viral diseases, in Nathanson N, Ahmed R, Gonzalez-Scarano F *et al.* (eds), *Viral pathogenesis*, Lippincott-Raven Publishers, Philadelphia, 1997, with permission.

Mouse population	Dead/total	Percent mortality
Inbred strains		
C3H/He	38/38	100
C3H/RV	0/34	0
Wild outbred mice		
Maryland	2/10	20
Soledad, California	0/5	0
La Puenta, California	0/5	0
Devonshire, California	0/5	0

TABLE 13.4 Different strains of mice differ in their susceptibility to flaviviruses. In this example, a few inbred and wild mouse populations were tested for their susceptibility to an attenuated yellow fever virus after intracerebral injection of 0.03 ml into adult animals
After Darnell MB, Koprowski H, Lagerspetz K. Genetically determined resistance to infection with group B arboviruses. I. Distribution of the resistance gene among various mouse populations and characteristics of gene expression in vivo. *Journal of Infectious Diseases* 1974, 129: 240–247, with permission.

A congenic resistant strain was developed, by repeatedly back crossing of virus-tested resistant F1 hybrids with susceptible C3H mice. The resistant congenic strain (C3H/RV) was compared with the susceptible C3H/He parental strain following infection with West Nile virus (WNV, a virulent flavivirus) (Table 13.4, Figure 13.3). After intraperitoneal injection of West Nile virus, 100% of susceptible animals – but no resistant animals – died. After intracerebral injection of WNV, virus replicates much less rapidly in the brains of resistant animals but sufficiently to kill some of them after an extended incubation. Resistance is not absolute and can be overcome by intracerebral inoculation of a virulent flavivirus, immunosuppression, or infection of newborn animals. However, in contrast to the Mx gene, interferon is not involved in expression of the resistance phenotype.

Cell cultures derived from the two strains of animals also differ in the efficiency with which they replicate flaviviruses and have been used to probe the cellular mechanism. West Nile virus could be readily maintained by serial passage in susceptible cells but disappeared after three passages in resistant cells. Flaviviruses are positive-stranded RNA viruses and are transcribed into negative-strand copies that are used as the templates for the genomes that are incorporated into nascent virions. Transcription of flavivirus RNA is less efficient in resistant cells, presumably because the *Flv* gene encodes a cellular protein that is involved either directly or indirectly in the regulation of viral transcription. Brinton and colleagues have recently identified the *Flv* gene as *Oas1b*, which encodes an inactive 2′-5′ oligoadenylate synthetase, but the mechanism of action of the full length Oasb1 gene product is not currently known. Susceptible mice encode a truncated version of Oas1b.

Inhibition of viral replication: the Fv1 gene and murine leukemia viruses (MuLV)

In the course of studies of the murine leukemia viruses, it was discovered that mouse cells could be divided into B type (named after BALB/c strain of mice) and N type (named after NIH strain of mice). MuLV isolates were either N-tropic or B-tropic depending upon which cells supported their replication. Cross-breeding of mice indicates that cellular susceptibility is determined by a single locus, named the *Fv1* locus, with two alleles, $Fv1^{n/n}$ or $Fv1^{b/b}$. $Fv^{b/b}$ cells are permissive for B-tropic viruses but resistant to N-tropic virus and vice versa, while $Fv1^{n/b}$ cells are resistant to infection with both N- and B-tropic

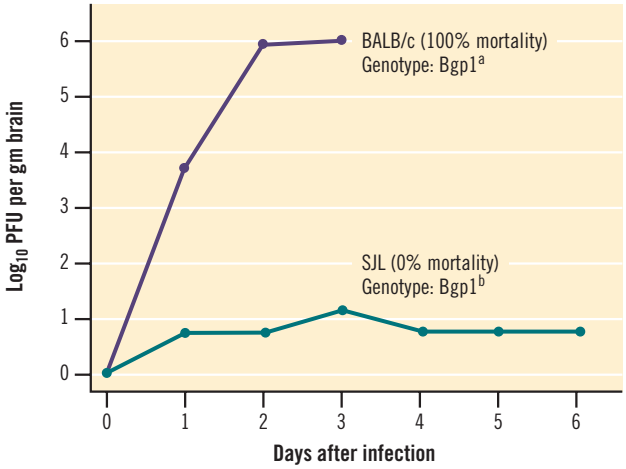

FIGURE 13.4 Pathogenesis of a viral infection in susceptible and resistant inbred strains of mice where susceptibility is determined by cellular receptors. Susceptible BALB/c mice are compared with resistant SJL mice after intracerebral injection of $10^{2.85}$ PFU of mouse hepatitis virus into adult animals. After Ohtsuka N, Taguchi F. Mouse susceptibility to mouse hepatitis virus infection is linked to viral receptor genotype. *Journal of Virology* 1997, 71: 8860–8863, with permission.

viruses. The *Fv1* locus encodes endogenous retroviral sequences that control a step in viral replication that occurs after entry and involves the CA (capsid) protein of the infecting virus. However, the nature of the interaction between the CA protein and the *Fv1* gene product remains to be determined.

Receptors: mouse hepatitis virus (MHV)

Differences in the susceptibility of inbred strains of mice to diseases caused by MHV (a coronavirus) was one of the first described examples of the genetic determinants mediating the outcome of a viral infection. Several distinct genetic loci have been identified in mice (see Table 13.2). This discussion is limited to the Hv-2 locus, mapped to chromosome 7, which influences the replication of MHV. Susceptible BALB/c mice can be infected and, depending upon host age, inoculation route and virus dose, killed by a number of different isolates of MHV, while SJL mice appear resistant to infection (Figure 13.4). Based on cross breeding of resistant and susceptible strains, susceptibility is determined by a single autosomal dominant genetic locus.

The receptor for MHV is a murine biliary glycoprotein (Bgp1), which is classified as a member of the immunoglobulin superfamily based on its structure as a transmembrane glycoprotein with four distinct globular domains. The Bgp1 gene has two allelic forms ($Bgp1^a$ and $Bgp1^b$) and these alleles cosegregate with the Hv-2 locus. Evidence that the Hv-2 locus encodes the viral receptor is provided by studies of F1 and F2 crosses of the resistant and susceptible strains, which show that there is a correspondence between Bgp 1 alleles and responses to MHV infection (Table 13.5).

Transforming retroviruses and their receptors

Different avian retroviruses (ASLVs) utilize different cellular membrane proteins as their receptors (see Figure 4.5 and Table 11.6) and this forms the basis for genetic differences between inbred strains of chickens in their susceptibility to a panel of ASLVs (discussed in Chapter 11).

Mouse strain	Bgp1 alleles	Mortality dead/total
BALB/c	a/a	33/33
SJL	b/b	0/27
F1 (BALB X SJL)	a/b	13/13
F2 (F1 X F1)	a/a	8/8
	a/b	23/23
	b/b	0/14

TABLE 13.5 Virus susceptibility may be determined by host-encoded cellular receptors. In this example, two inbred strains of mice (BALB/C and SJL) encode different variants of the MHVR (mouse hepatitis virus receptor), designated $Bgp1^a$ and $Bgp1^b$. These experiments show that mice expressing $Bgp1^a$ are susceptible and illustrates segregation of the MHV susceptibility as a single genetic locus, where susceptibility is dominant. The genotypes of individual mice were determined by single strand conformation polymorphism that exploits the different migration on polyacrylamide gels of PCR fragments of DNA of the $Bgp1^a$ and $Bgp1^b$ genotypes. Adult mice were infected with $10^{7.85}$ of the JHM sp-4 strain of MHV and mortality determined two weeks later
After Ohtsuka N, Taguchi F. Mouse susceptibility to mouse hepatitis virus infection is linked to viral receptor genotype. *Journal of Virology* 1997, 71: 8860–8863, with permission.

Immunological determinants

Lymphocytic choriomeningitis virus (LCMV)

LCMV is a virus whose persistence and disease induction is dependent upon its ability to induce an immune response mediated by CD8+ cytolytic T lymphocytes (CTLs). Immunogenetic differences between mouse strains have been associated with variations in the host response to LCMV infection. Figure 13.5 compares two mouse strains that vary in their cellular immune responses to LCMV. Animals that have a deletion in one locus within the MHC region of their genomes exhibit a reduced immune response to LCMV infection and become persistently infected, while congenic normal mice mount a brisk immune response and clear the virus.

Murine leukemia virus (MuLV)

Several MHC-associated genes influence the outcome of infection with murine leukemia viruses (see Chapter 11 for background information). These include the *Rfv-1* and *Rfv-2* genes that affect recovery from Friend (F) MuLV-induced erythroleukemia and the *Rgv-1* gene that affects recovery from Gross (G) MuLV-induced leukemia. Although the murine leukemia virus model is a somewhat contrived one, based on laboratory derived retroviruses and inbred strains of mice, it illustrates genetic determinants that act through the immune response. Figure 13.6 shows an example of host differences in the development of leukemia induced by F-MuLV that map to discrete regions within the H-2 (MHC).

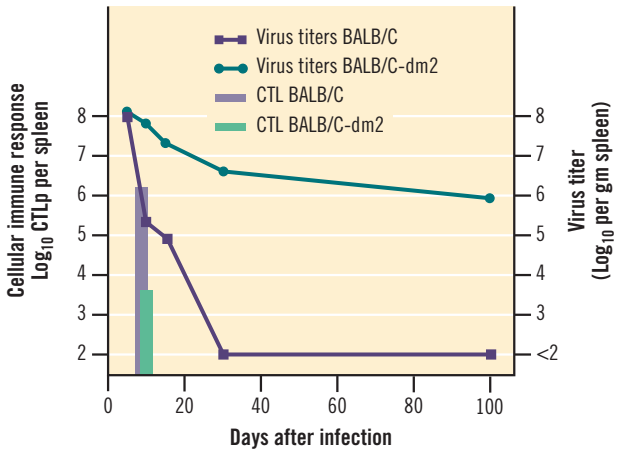

FIGURE 13.5 Some genetic determinants of the host response to infection are associated with immune responses and map to the major histocompatibility complex (MHC). In this example, two inbred strains of mice differing only in one region of the H-2 region (mouse MHC) are compared after infection with 10^2 PFU of the docile strain of LCMV (lymphocytic choriomeningitis virus). BALB/c and BALB/c-dm2 mice are H-2^d (d haplotype) but the dm2 mice are mutants lacking the L region of the H-2 locus. The dm2 mice are poor immunological responders to LCMV and are persistently infected, in comparison to BALB/c mice that mount a brisk immune response and clear the virus. After Moskophidis D, Lechner F, Hengartner H, Zinkernagel RM. MHC Class I and non-MHC-linked capacity for generating an anti-viral CTL response determines susceptibility to CTL exhaustion and establishment of virus persistence in mice. *Journal of Immunology* 1994, 152: 4976–4983, with permission.

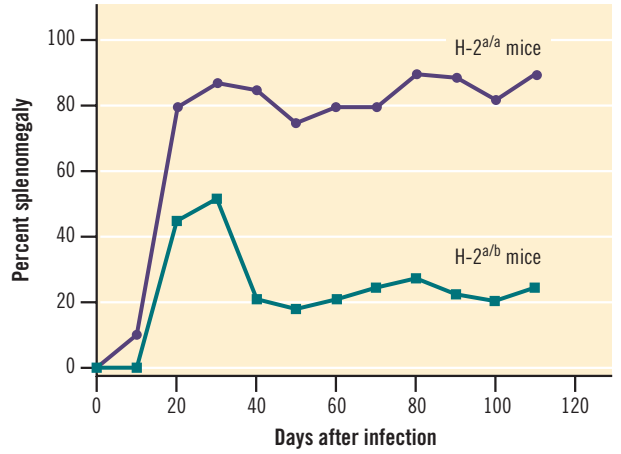

FIGURE 13.6 An example of host genetic determinants that map to the MHC. Two congenic strains of mice, which differ only at the H-2 locus (H-$2^{a/a}$ or H-$2^{a/b}$), were injected with 15 FFU (focus forming units) of F-MuLV (Friend virus strain of murine leukemia virus) and followed for the development of splenomegaly or death, which are signs of erythroleukemia caused by the virus. The resistant H-$2^{a/b}$ mice controlled their leukemia and survived, while the H-$2^{a/a}$ mice did not control their leukemia and died. Recombinant strains between the a and b haplotypes were used to show that there were two genetic loci involved, the Rfv-1 locus in the D-L region of H-2 and the class II locus in the KI-A region of H-2. It appears that the Rfv locus acts via the CD8 response to viral antigens and the KI-A locus influences the activity of virus-specific CD4 helper cells. After Miyazawa, M, Nishio J, Wehrly K, Chesebro B. Influence of MHC genes on spontaneous recovery from Friend retrovirus-induced leukemia. *Journal of Immunology* 1992, 148: 644–647, with permission.

West Nile virus (WNV) and CCR5

It has recently been reported that CCR5, a chemokine receptor (and the coreceptor for many strains of HIV-1), plays a critical role in the survival of mice from experimental infection with WNV. Apparently WNV infection leads to central nervous system upregulation of cellular ligands for CCR5, thereby enhancing the migration of macrophages and T lymphocytes to the nervous system, where they provide a host defense against ongoing WNV infection. Furthermore, human patients with symptomatic WNV infection showed a higher frequency of the Δ32 mutation ($CCR5^{-/-}$) compared with normal control subjects.

GENETIC DETERMINANTS IN HUMANS

HIV/AIDS

The intense investigation of HIV has provided evidence of host determinants of a human viral disease.

Resistance to HIV infection

Epidemiological studies indicate that there is significant variation in human susceptibility to HIV infection. Thus, some persons fail to develop the markers of infection (serum antibody and evidence of HIV RNA or DNA in blood) in spite of repeated sexual exposure to infected contacts. There appear to be at least two different mechanisms for resistance to infection, the absence of the viral coreceptor and local immunity.

As described in Chapter 3, the entry of HIV into susceptible human cells involves a primary receptor (CD4) and a coreceptor (CCR5 or CXCR4, both chemokine receptors). Some humans have a natural mutation in the CCR5 gene that abrogates expression of the CCR5 protein (the Δ32 mutation) and individuals who are homozygous for Δ32 are resistant to infection with the majority of wildtype strains of HIV-1, since most of these utilize CCR5 but not CXCR4 as a coreceptor. In the Caucasian population of the USA, the relative allelic frequencies for CCR5 are about: −/− individuals, 1%; +/+ individuals, 81%; and +/− individuals, 18%. When an HIV at-risk cohort was divided into infected and uninfected groups, the HIV-infected group contained no individuals who were homozygous for the Δ32 mutation while the HIV-negative group had an excess (above expectancy) of individuals with the Δ32 mutation (Table 13.6). Recently, it has been shown that there are gene duplications for CCL3L1, a chemokine that is a ligand for CCR5. Within human populations, there is considerable variation in the number of duplications and a large number of gene duplications reduces risk of infection and slows progression to AIDS. Presumably, CCL3L1 reduces the availability of CCR5 on the cell surface by acting as a competitor for HIV infection.

Among subjects at very high risk of HIV infection because of their life style (for instance, commercial sex workers), a very small proportion (<5%) remain uninfected. Such subjects are called EU (exposed uninfected)

HIV-1 antibody	Parameter	Total	CCR5 +/+	CCR5 +/−	CCR5 −/−
Positive	Number	1343	1148	195	0
	Percent	100	85	15	0
Negative	Number	612	508	87	17
	Percent	100	83	14	3

TABLE 13.6 Differences in host susceptibility have been defined for a few viruses of humans. The chemokine receptor CCR5 acts as a coreceptor for most wildtype HIV isolates. A naturally occurring mutation (Δ32) in the CCR5 gene abrogates the expression of CCR5 and homozygous carriers of this mutation are resistant to HIV-1. Subjects at high risk of HIV exposure were divided into those who were HIV-infected and those who were not and tested for their CCR5 genotype (CCR5 +/+, +/−, or −/−). There was a striking absence of $CCR5^{-/-}$ persons in the HIV-infected group and an excess of $CCR5^{-/-}$ persons (above the 1% expectancy) in the HIV-uninfected group After Dean M, Carrington M, Winkler C *et al.* Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. *Science* 1996, 273: 1856–1862, with permission.

or ESN (exposed seronegative). There is some evidence that these persons have developed local immunity in the genital tract, since they may demonstrate either HIV-specific CTL activity in their peripheral blood lymphocytes or IgA antibody in their genital secretions. It remains to be determined whether a host factor or the circumstances of exposure explain ESN status.

Resistance to AIDS

Among HIV-infected persons, there are marked differences in the rate of progression to AIDS, which can vary from about 1 year to >20 years. Many factors influence incubation period, including the pathogenicity of the virus, the dose and route of infection and the exposure to opportunistic infections. The potential role of host determinants in this variability has yet to be completely analyzed, but one factor is CCR5. Thus, patients who are heterozygous for the Δ32 mutation ($CCR5^{+/-}$) progress more slowly to AIDS than do those homozygous for the coreceptor ($CCR5^{+/+}$). Age is another factor, since, in the absence of anti-retroviral therapy, median survival time (HIV infection to death) is <2 years in infants and >10 years in adults.

It is likely that there are other significant host variables associated with HIV infection, a hypothesis suggested by observations on SIV (simian immunodeficiency virus). SIV produces AIDS in monkeys and is an excellent model for HIV-induced AIDS in humans. There are dramatic differences between the levels of viremia in individual SIV-infected monkeys and these correlate with the length of the incubation period prior to clinical AIDS. Furthermore, in vivo susceptibility is mirrored in the levels of SIV replication in peripheral blood mononuclear cells (PBMCs) (Figure 13.7). Since these differences occur in monkeys of similar age that are homozygous for wild-type CCR5, additional host factors must be operative.

Immunological determinants of AIDS

In addition to the host factors described above, HLA-associated determinants influence the progression of HIV infections. Among HIV-infected patients, those that remain normal for the longest period are often called long-term non-progressors. One comparison of

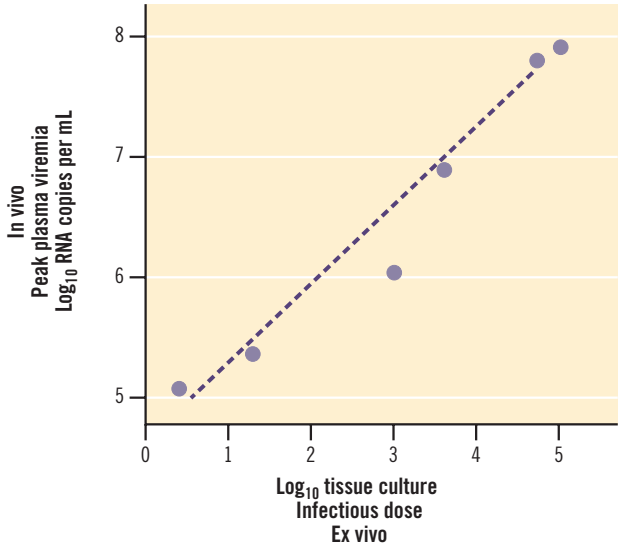

FIGURE 13.7 Host variability in SIV infection of rhesus monkeys, which is an excellent model for HIV infection of humans. Using a standard virus inoculum (cloned pathogenic SIVsmE543–3), the course of infection in individual monkeys was compared both in vivo and ex vivo. This figure shows data from six animals that were selected for their variable susceptibility, indicating that there was a consistent relationship between the peak titer of virus in PBMCs (ex vivo) and peak viremia (in vivo). After Goldstein S, Brown CR, Dehghani H, Lifson JD, Hirsch VM. Intrinsic susceptibility of rhesus macaque peripheral CD4+ T cells to simian immunodeficiency virus in vitro is predictive of in vivo viral replication. *Journal of Virology* 2000, 74: 9388–9395, with permission.

non-progressors with progressors shows an over-representation of a single HLA haplotype (B-57), suggesting that immunogenetic determinants can influence susceptibility to AIDS (Table 13.7). Several HLA haplotypes have been associated with differences in progression to AIDS among HIV-infected patients and may influence differences in the breadth and intensity of the antiviral cellular immune response (Table 13.8). Among the three class I HLA loci (HLA-A, -B, -C), HLA-B-restricted epitopes are the most important determinants of protective CD8-specific responses.

Other human viruses

Although data on genetic determinants of susceptibility to other human viruses are sparse, it appears likely that

Status of HIV-infected patients	Frequency of B57 HLA haplotype	
	B57/total	Percent B57
Non-progressors	11/13	85
Progressors	19/200	10

TABLE 13.7 HLA-associated determinants may influence the course of HIV infection. In this example, the HLA haplotypes of two groups of HIV-infected persons were compared, long-term non-progressors and progressors. A single HLA haplotype, B57, was over–represented in the non-progressor group, while in the progressor group the B57 haplotype occurred at a frequency similar to that seen in the Caucasion population of the USA. This implies that persons with B57 produce a more protective immune response to HIV than do individuals with other haplotypes, but the exact mechanism remains to be established
After Migueles SA, Sabbaghian MS, Shupert WL *et al.* HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proceedings of the National Academy of Sciences* 2000, 97: 2709–2714, with permission.

genetic determinants influence the outcome of most viral diseases. Recent large scale studies have identified immunogenetic alleles that influence the course of several persistent viral infections, such as those caused by HPV, HBV and HCV.

HLA genotype	Effect on course of HIV infection	Possible functional explanation	Genotype
Class I homozygosity (1, 2 or 3 loci)	Rapid progression	Narrow range of T cell epitopes	
HLA-B*27	Slow progression	Immunodominant conserved epitope under structural constraint	Dominant
HLA-B*57	Slow progression	Broad cross-reactivity against HIV peptides	Dominant
HLA-B*35	Rapid progression	Unknown	Co-dominant
HLA mismatch between HIV donor and recipient	Reduces risk of infection	Allogeneic response against infected donor cells	

TABLE 13.8 HLA haplotypes that influence the course of HIV infection
After Carrington M, O'Brien SJ. The influence of HLA genotype on AIDS. *Annual Review of Medicine* 2003, 54: 535–551.

PHYSIOLOGICAL FACTORS

Age

Infancy

The effects of immaturity have been studied in detail in experimental murine models, since the maturation process, from birth to adulthood, is completed in about two months and it is easy to obtain rodents of precisely documented age. Several generalizations can be made from these studies (Sidebar 13.1).

Most viruses are more lethal in newborn than in adult animals, which usually reflects differences in the extent of replication (Figure 13.8). Such distinctions are apparent within the first 24–48 hours of infection, implying that they are not due to acquired immunity. However, they may reflect innate host defenses, such as the induction of interferon. Age-specific reduction in susceptibility is often associated with differentiation of cells. For instance, if multipotent chromaffin cells are treated with nerve growth factor that causes them to differentiate, there is a marked reduction in the production of LCMV and this is reversed when the factor is removed. Likewise, if young rats are transplanted with embryonic brain tissue and challenged with Japanese encephalitis virus, replication is restricted to the transplant reflecting the higher susceptibility of embryonic versus infantile neurons.

Newborn mice are immunologically immature and, depending upon the specific virus, evolve to reach an 'adult' immune response at 1–3 months of age. For viruses that produce an immune-mediated illness, newborn animals may be 'tolerized' by the same virus inoculum that induces disease in adult animals (see Chapter 8). By the same token, persistent viruses are more likely to initiate a lifelong infection in newborn than in adult mice, which usually correlates with tolerance following infection of newborn animals.

SIDEBAR 13.1

Age-specific differences in susceptibility to viral infection

- Most viruses are more lethal in newborn than in adult animals
- Differences in susceptibility usually reflect the extent of viral replication
- In some instances, age influences intracellular responses to viral infection
- Immunological immaturity explains some instances of increased susceptibility in infants

Human infants are also more susceptible to diseases induced by many viruses, as shown in Table 13.9. Since infants acquire passive antibody from their mothers, both across the placenta and via breast feeding, they are often protected during the first 3–24 months of life. If a human population has not been exposed to a given virus, both mothers and their infants lack immunity and the influence of age on susceptibility is revealed. Such a situation occurred in 1846 in the Faroe Islands, isolated in the north Atlantic Ocean, during an epidemic of measles. Since the last recorded outbreak of measles in the Faroe Islands had taken place 75 years previously, most of the population was non-immune. Under these circumstances, there was a particularly high mortality in infants, illustrating their biological vulnerability due to the absence of immune protection. This example also provides a teleological explanation for the evolutionary development of passive transfer of immunity from mother to infant.

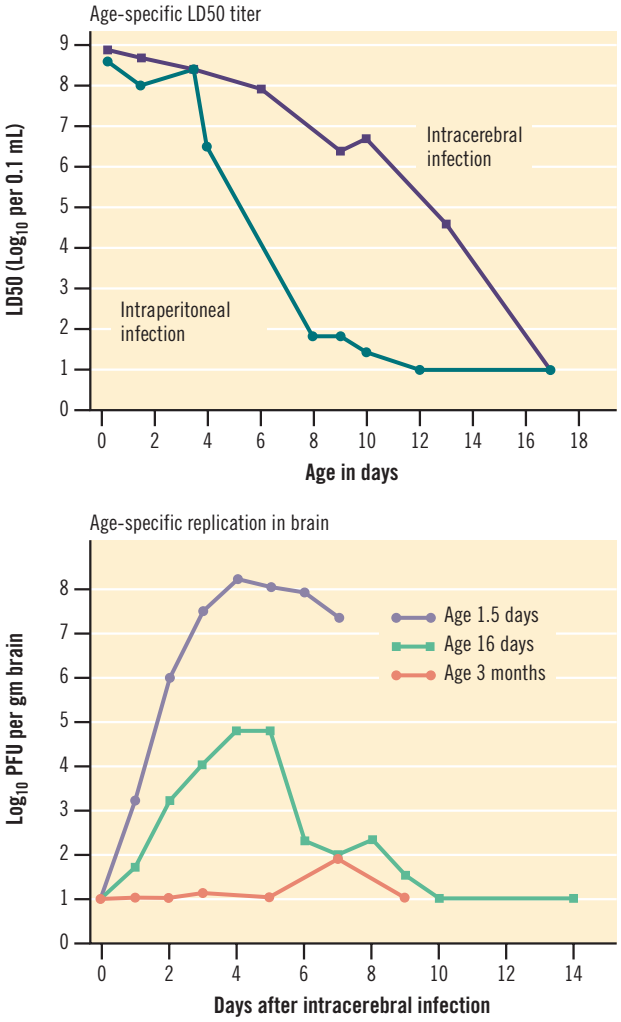

FIGURE 13.8 Newborn animals are usually much more susceptible to viral infection and disease than are their adult counterparts. In this example, West Nile virus, a flavivirus, was used to infect Wistar rats. Upper panel: age-specific titration by intracerebral and intraperitoneal routes. For each age group, serial dilutions of the virus were injected (0.1 ml per animal) and the LD50 (50% lethal dose) titer was determined. Although highly susceptible at birth, rats developed resistance to disease by two weeks of age. Lower panel: susceptibility reflects the extent of virus replication, as shown by the replication of WNV in the brain following intracerebral injection of rats 1–2 day-old animals (3 PFU inoculum) or older ($10^{5.1}$ PFU). After El Dadah AH, Nathanson N, Sarsitis R. Pathogenesis of West Nile virus encephalitis in mice and rats. *American Journal of Epidemiology* 1967, 86: 765–790, with permission.

Age group	Annual mortality (all causes) per 100		
	1835–1845	1846	Excess in 1846
<1	10.8	30.0	19.2
1–9	0.5	0.5	—
10–19	0.5	0.5	—
20–29	0.5	0.7	0.2
30–39	0.8	2.1	1.3
40–49	1.1	2.7	1.6
50–59	0.9	4.4	3.5
60–69	2.0	7.7	5.7
70–79	6.5	13.1	6.6
80–100	16.8	26.0	9.2

TABLE 13.9 Increased susceptibility of human infants to viral disease as exemplified by measles. This table shows age-specific differences in mortality from the measles epidemic of 1846 in the Faroe Islands, compared with average mortality for 1835–1845. The excess mortality for 1846 provides a crude estimate of measles-specific mortality during the epidemic, which involved at least 75% of the population. The data demonstrate a dramatic increase in infant mortality and also suggest that there was an increase in mortality in the older age groups
Data from Panum PL. *Observations made during the epidemic of measles on the Faroe Islands in the year 1846*, American Public Health Association, New York, 1940, with permission.

For a number of other virus diseases, such as Western equine encephalitis, human immunodeficiency, herpes simplex and smallpox viruses, it has been shown or inferred that infants are more susceptible than adults. On the other hand, there are a few viruses where infection of infants induces immune tolerance and a persistent infection, in contrast to the acute primary infection of adults characterized by an immune response that both clears the virus and causes disease. The most prominent example of this paradigm is HBV, as described in Chapter 8. Figure 8.9 illustrates the tolerizing effect of rubella virus infection in utero compared to an immunizing infection in childhood.

In a few instances, maturation increases susceptibility to specific viruses. For instance, intracerebral injection of a rodent-adapted poliovirus strain paralyzed older mice more rapidly and at a greater frequency than newborn mice. The difference was due to the failure of virus to travel along neuronal pathways from brain to spinal cord in newborn mice, since both newborn and 4-week-old mice were equally susceptible to intraspinal poliovirus injection. It was hypothesized that maturation of the fast axonal transport system – that could move poliovirus nucleocapsids from brain to cord – might account for this paradoxical finding.

Cellular mechanisms of age-specific susceptibility

In some instances, age influences intracellular responses to viral infection. For instance, bcl-2, an anti-apoptosis protein, protects mouse neurons against the lethal consequences of infection with Sindbis, an alphavirus; as mice mature, bcl-2 expression increases and is thought to explain age-specific development of resistance against Sindbis mortality. Selected Sindbis virus mutants can overcome age-specific resistance and this property has been mapped to a single amino acid in one of the envelope proteins.

The parvoviruses, small DNA viruses that can only replicate in dividing cells, provide another example. Proliferation of specific cell types is an essential part of normal development. External granule cells of the rodent cerebellum divide through the first postnatal week, after

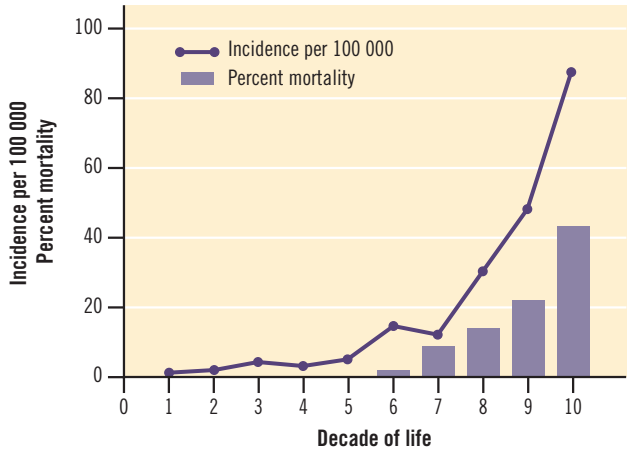

FIGURE 13.9 Older age increases disease susceptibility for some viruses, as shown for West Nile virus (WNV), a flavivirus. The figure shows the age-specific disease incidence per 100 000 population and percent mortality (among confirmed cases) for an outbreak of more than 400 cases of West Nile fever in Israel in 2000. After Weinberger M, Pitlik SD, Gandacu D *et al.* West Nile fever outbreak, Israel, 2000: epidemiologic aspects. *Emerging Infectious Diseases* 2001, 7: 686–691, with permission.

which they migrate to form the internal granule cell layer. If rats are infected with rat virus, a murine parvovirus, shortly after birth, the virus destroys the granule cells leading to severe atrophy of the cerebellum, while infection of weanling animals (age three weeks) is inapparent.

Old age

A few viruses appear to cause more severe disease in older persons and Figure 13.9 illustrates this phenomenon for West Nile virus. It has been speculated that in older persons localized gaps in the blood–brain barrier increase the risk that virus can pass from blood into the central nervous system, the target organ for encephalitic viruses. Also, immune responses wane in old age and may contribute to increased severity of certain viral diseases. For instance, herpes zoster (a reactivation of VZV infection – chickenpox – acquired in childhood) is much more frequent above age 70 and measles appears to be more severe in older persons (see Table 13.9).

Gender

There are gender-specific differences in the outcome of certain viral infections as suggested by the data in Table 13.1. The relative male-female prevalence of sexually transmitted viral infections of humans is often unequal, as has been documented for herpes simplex type 2 and HIV. A prophylactic HSV 2 vaccine was partly protective in women but not in men, again suggesting gender-specific differences in transmission and pathogenesis. Furthermore, the use of hormonal contraception by women clearly alters their susceptibility to some sexually transmitted viruses, such as HIV. Such differences undoubtedly reflect both biological factors such as variation in the route of infection and gender-specific behavioral determinants.

Other determinants of susceptibility

There are limited quantitative data on other host determinants of infection. Conditions and diseases that are associated with immunodeficiency, such as tissue transplantation and AIDS, can enhance host susceptibility to viral infections. Severe undernutrition can alter (often enhance) the severity of viral disease, both in animals and in humans and this may be due to compromise of immune responses. Also, pregnancy has an adverse effect on the prognosis of some viral infections such as hepatitis B, hepatitis E and poliomyelitis.

REPRISE

The availability of a large number of inbred genetically uniform strains of mice has permitted the identification of ~25 host genes that influence susceptibility to viral infection. Most of these determinants are specific for a single virus or virus family and many are involved in a step in the viral replication cycle. A smaller number of determinants map within the MHC complex and affect the immune response. Genetic determinants similar to those identified in mice probably operate in many species, but are difficult to ascertain in outbred, free-living populations. However, an increasing number of specific human genes that affect the outcome of viral infections are being identified, particularly in the MHC locus and in loci that influence innate immunity. Some of the susceptibility/resistance genes have been sequenced and the normal functions of their products determined. The molecular mechanisms whereby different allelic variants affect viral infection or disease outcome are incompletely understood and there are a great many relevant genes that have yet to be identified. In addition to genetic determinants, many other host variables, such as age, gender, nutritional status and other physiological variables influence the outcome of a viral infection. Analysis of relevant mechanisms is very incomplete and represents a fertile area for future investigation.

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Chapter 14

HIV, SIV and the Pathogenesis of AIDS

Neal Nathanson and Julie Overbaugh

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INTRODUCTION

At the turn of the millennium, HIV is probably the single most significant human viral infection. Furthermore, as a complex and enigmatic example of viral pathogenesis, it has been the subject of more intensive study than any other viral disease. This has led to a dynamic analysis of virus–host interactions, a pioneering achievement that supplements established approaches to pathogenesis. For these several reasons, a full chapter has been devoted to HIV and AIDS, including studies of simian models.

The chapter begins with a description of virus replication, cellular responses and the sequence of viral, immunological and disease events that follow HIV infection. It then turns to the dynamic aspects of infection, including the turnover of virus and viral latency, the turnover of cellular populations and the evolution of viral phenotypes. Finally, data are synthesized to explain the pathophysiology of immunodeficiency, opportunistic infections and HIV-associated neoplasms. Prophylactic vaccines for HIV are discussed in Chapter 17.

Our understanding of the pathogenesis of AIDS is based on clinical studies in humans infected with HIV and on experimental studies in monkeys infected with SIV (simian immunodeficiency virus). The SIVs are relevant since some of them cause AIDS in non-human primates and have contributed important insights that could not be gleaned from human studies alone. However, it is important to be aware that, in general, the monkey model has been deliberately manipulated to cause a high incidence of clinical AIDS with a short incubation period. Therefore, studies with SIV must be interpreted with caution when they are extrapolated to reconstruct the pathogenesis of AIDS in humans.

LENTIVIRUSES

HIV is a member of the lentiviruses. In common with all retroviruses, lentiviruses have three major genetic loci (*gag, pol, env*) that encode the core proteins, the reverse transcriptase and integrase and the envelope

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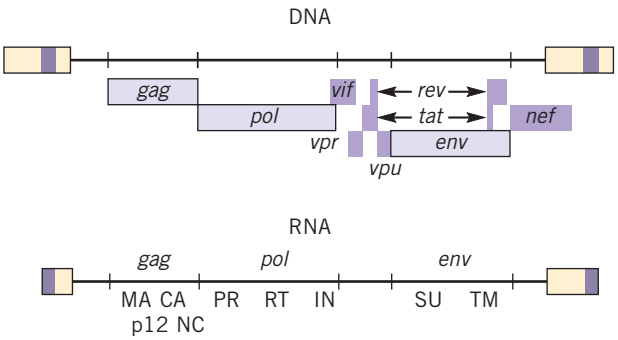

FIGURE 14.1 Organization of the HIV-1 genome. The RNA genome is about 9 kb long and is bounded at both ends by a non-coding repeat (R) region that encloses the three major coding genes, the *gag* (group antigen), *pol* (polymerase) and *env* (envelope) genes that are transcribed in three different reading frames. The diagram indicates the position of the major proteins encoded by each of the genes, including the MA (matrix), p12, CA (capsid), NC (nucleocapsid) proteins of gag, the PR (protease), RT (reverse transcriptase), IN (integrase) enzymes of pol and the SU (surface) and TM (transmembrane) proteins of env. HIV-1 also encodes six non-structural accessory proteins (tat, rev, nef, vpr, vpu, vif) whose open reading frames are shown.

proteins, respectively (see Figure 11.1). HIV is distinguished from the non-lenti retroviruses by several characteristics:

1. HIV possesses six accessory genes that encode non-structural proteins. Two of these genes (*tat* and *rev*) are required for replication in cell cultures. Four (*vpr, vpu, vif, nef*) accessory genes are not absolutely necessary for replication in cell culture systems but at least some (such as *nef*) are required for full virulence in vivo (Figure 14.1).
2. The viral attachment protein binds to the CD4 molecule that is found on the CD4+ subset of T lymphocytes and monocytoid cells (macrophages, microglia and dendritic cells) and this determines its cellular host range.
3. HIV can replicate in non-dividing (as well as dividing) cells in contrast to other retroviruses that replicate only in dividing cells. In the case of lentiviruses, the pre-integration complex of reverse transcribed viral DNA and proteins can be imported across the nuclear envelope, permitting infection of non-dividing cells.
4. HIV causes lifelong infections that are associated with a number of chronic diseases, including AIDS, but they do not encode oncogenes.
5. HIV is a strictly exogenous virus and host genomes do not include copies of HIV sequences.

The lentivirus family includes members that infect horses, cows, sheep, goats, cats and non-human primates. There are two major groups of human lentiviruses, HIV-1 and HIV-2, both of which represent viruses that crossed the species barrier from non-human primates. HIV-1 was most likely derived from chimpanzee viruses (SIVcpz) and HIV-2 from sooty mangabey viruses (SIVsm), both primate species that are found only in Africa. HIV-1 can be classified in three subgroups, M (main), N (new) and O (outlier) and the M subgroup (that is most common) can be divided into nine genotypes also called clades (A–D, F–H, J, K). This chapter is based mainly on studies of HIV-1 rather than HIV-2, because HIV-1 has been the focus of most research.

Many SIV strains cause lifelong persistent non-pathogenic infections in the species in which they are enzootic. However, if an SIV strain is transmitted to a non-enzootic simian host it may cause AIDS. Thus, there are several laboratory-passaged strains of SIV, probably derived originally from SIVsm, which are pathogenic in rhesus monkeys (Asiatic monkeys never exposed to SIV sm from Africa). Likewise, untreated HIV-1 is ~100% lethal in humans, but is relatively non-pathogenic when used for experimental infection of chimpanzees, the species from which it probably originated. These phenomena carry important implications regarding the mechanisms by which HIV causes AIDS (discussed below).

VIRUS–CELL INTERACTIONS

Receptors and viral tropism

The entry of HIV into permissive host cells is a multistep process (Figure 14.2) that involves a primary receptor and a coreceptor. In all cases the primary receptor is CD4, an immunoglobulin superfamily molecule expressed on two major cell types, the CD4+ subset of T lymphocytes and cells of the monocyte lineage. The coreceptor is one of several members of the chemokine receptor family of molecules, particularly CCR5 and CXCR4. CCR5 is expressed on CD4+ T lymphocytes and macrophages but not on T cell lines (TCL), while CXCR4 is expressed on CD4+ T lymphocytes, on TCLs and, at very low concentrations, on macrophages.

Initially, the viral attachment protein (SU protein, or gp120) binds to CD4, which triggers a conformational change that leads to binding of SU to the coreceptor. Binding to the coreceptor triggers a second conformational change in the TM (transmembrane or gp41) protein that is often described as releasing a spring, resulting in the approximation of the N terminal domain of the TM protein to the plasma membrane of the cell. A hydrophobic fusion peptide at the N terminal domain of the TM protein inserts into the plasma membrane, forming a pre-hairpin configuration. A third conformational change in the TM protein closes the hairpin, leading to fusion between the viral envelope and the cellular membrane.

HIV-1 strains vary in their ability to bind to coreceptors and this affects their cellular host range (see Chapter 3). Viruses isolated from patients may be roughly classified into three groups:

1. those that utilize mainly CCR5 (often called R5 viruses)
2. those that utilize mainly CXCR4 (X4 viruses)
3. those that utilize both CCR5 and CXCR4 (R5X4, or 'dual-tropic' viruses).

(In early studies of HIV-1, the amplification of virus in T-cell lines led to the creation of T-cell-adapted viruses

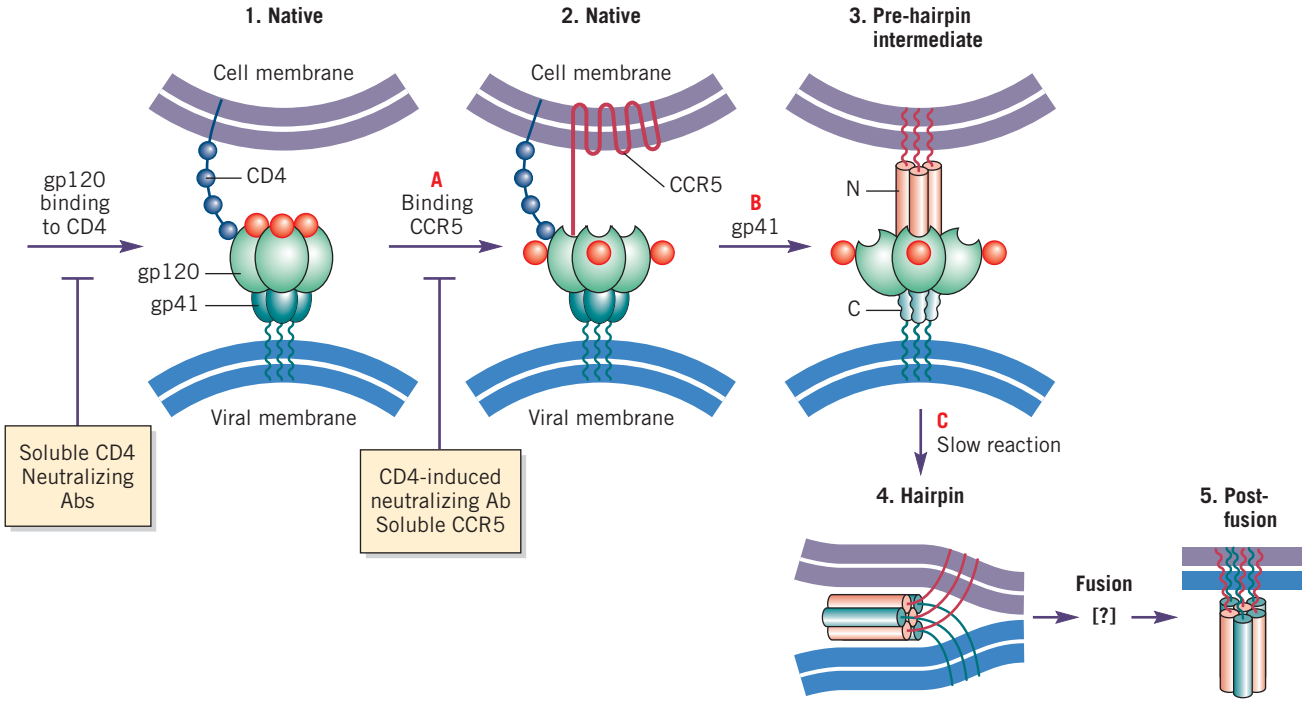

FIGURE 14.2 Entry of HIV showing a hypothetical reconstruction of stepwise conformational changes in the SU (gp120) and TM (gp41) proteins. The gp120 protein binds to the CD4 receptor. Binding to CD4 triggers a conformational change (A) in the gp120 that permits it to bind to the coreceptor (CCR5). This leads to a second major conformational change (B), this one in the TM (gp41) protein, which unfolds to expose and insert the fusion sequence at its N terminus into the plasma membrane of the cell, producing the pre-hairpin intermediate. In a third conformational change (C), helices in the N and C domains of gp41 associate, producing the hairpin configuration that brings the viral envelope and the plasma membrane into close approximation. Finally, the two membranes fuse, leaving gp41 on the external surface. After Chan DC, Kim PS. HIV entry and its inhibition. *Cell* 1998, 93: 681–684, with permission.

(X4 viruses) that differ in some of their biological properties from fresh human isolates and are rarely used in current research.)

All HIV-1 strains can replicate in primary T lymphocyte cultures that express both CCR5 and CXCR4. Among isolates grown in these cells, the R5 and R5X4 viruses can infect cultured macrophages and, contrary to earlier views, some of the wildtype X4 viruses can also infect macrophages. By contrast, the X4 and R5X4 (but not the R5) viruses can replicate in T cell lines. Finally, it is important to note that coreceptor expression is not a completely reliable predictor of cellular host range and that cell culture studies of coreceptor utilization may not necessarily reflect in vivo tropism.

HIV replication and host cell response

HIV-1 replicates slowly in permissive cells, relative to many other viruses. If the infected lymphocyte is actively dividing, then viral replication proceeds at a maximal rate. If the infected T cell is resting, the provirus can enter the nucleus and integrate, but usually remains latent until the T cell begins to divide, when HIV replication proceeds. Recent data suggest that HIV may also replicate at low levels in some resting T cells.

Various differentiated cell types – circulating monocytes, tissue macrophages, brain microglia and dendritic cells of the skin and central lymphoid tissue – are derived from monocyte precursors in the bone marrow. Each of these cells plays a role in the pathogenesis of HIV infection and some are permissive for HIV. HIV replicates in cultured monocyte-derived macrophages, although they produce lower titers of infectious virus than do proliferating CD4+ T lymphocytes.

Mature dendritic cells (DCs, see Chapter 5) are of particular importance in vivo, because they bind, sequester and conserve infectious virions at their extracellular surface or within endosomes, although they are poorly (or non-) permissive for virus replication. In contrast to mature DCs, immature DCs express CCR5 and support low levels of HIV replication. A lectin molecule, DC-SIGN that is expressed on the surface of dendritic cells, has been implicated in the binding and trapping of HIV particles. Virus captured by dendritic cells at mucosal sites can be carried to draining lymph nodes where it may be passed to permissive CD4+ T lymphocytes, particularly at the immunological synapse (the interface between DCs and T cells).

Cell killing in vitro

HIV varies in its ability to cause cell killing in permissive cells, depending both on the cell type and the viral strain used for infection. Most HIV strains replicate well in primary T lymphocyte cultures, but they vary in their cytopathic activity, which often correlates with replicative capacity, syncytium induction and coreceptor utilization. HIV replication in macrophage cultures is typically low relative to lymphocyte cultures and the cultures remain viable for many weeks.

Species source of TRIM5α		Degree of infection with HIV-1-GFP(%)	Degree of infection with SIV-GFP (%)
Human	Hominoid	18	20
Chimpanzee		19	18
Orang-utan		21	27
Rhesus	Old World monkeys	2	10
African green monkey		4	25
Squirrel monkey	New World monkeys	50	2
Tamarind		23	1
Spider monkey		1	1

TABLE 14.1 TRIM5α-mediated restriction of primate lentiviruses. HeLa cells transduced with vectors expressing TRIM5α from various species were infected with HIV-1 or SIV recombinant viruses expressing green fluorescent protein (GFP) and the per cent of infected cells that expressed GFP after exposure to virus was counted
After Song B, Javanbakht H, Perron M, Park DH, Stremlau M, Sodroski J. Retrovirus restriction by TRIM5α variants from Old World and New World primates. *Journal of Virology* 2005, 79: 3930–3937, with permission.

HIV or SIV can destroy CD4+ lymphocytes by several mechanisms. CD4+ lymphocytes vary in their susceptibility to infection and therefore in their vulnerability, depending upon their physiological status. Activated proliferating CD4+ cells are much more susceptible than resting cells and certain subpopulations of activated cells may be particularly susceptible, depending partly on the levels of X4 or R5 coreceptors expressed, which can vary widely. Single-cell killing is due to apoptosis, initiated in part by the tat protein and mediated through Fas signaling and the caspase cascade (see Chapter 4). Alternatively, infection of contiguous cells often results in syncytium formation and cell death.

Cell killing in vivo

In addition to the direct virus-induced cytopathic effects seen in cell cultures, immune-mediated destruction of infected cells occurs in vivo. HIV-infected patients mount a cellular immune response in which CD8 lymphocytes, through their T cell receptors, recognize and lyse infected (and perhaps uninfected) cells that present viral peptides in the context of class I MHC (HLA) antigens (see below). Also, HIV appears preferentially to infect the subset of CD4+ lymphocytes that are responding to HIV antigens, perhaps because HIV-specific T lymphocytes are rapidly dividing during immune induction, making them a vulnerable target for HIV-mediated infection and killing.

In vivo, during clinical latency, only a small minority (<5%) of CD4+ lymphocytes are infected at any time, which suggests that, in addition to destruction of infected CD4+ positive cells, other mechanisms probably play a role in CD4+ lymphocyte depletion. HIV or SIV infection causes a generalized activation of all lymphocyte populations (CD4+, CD8+, NK, and B cells) and a high proportion of activated cells undergo rapid apoptosis. Furthermore, HIV infection may interfere with the regeneration of CD4+ lymphocyte populations either in the bone marrow or in the thymus (pathogenic mechanisms are discussed later in this chapter).

Intrinsic cellular defenses

In the last 5 years, a number of 'intrinsic' cellular defenses against specific viruses have been uncovered (discussed in Chapters 4, 5 and 16). These defenses differ from both innate immunity and acquired immunity and most involve cellular molecules that can block or interfere with individual steps in the replication of specific viruses. Several cellular defenses against HIV have been discovered and two that have been the topic of intensive study are described briefly.

Post-viral entry restriction: TRIM5α

HIV-1 efficiently infects human cells but will not replicate in the cells of Old World monkeys (Table 14.1). The restrictive cells express the CD4 receptor and one of the coreceptors (CCR5 or CXCR4) and HIV-1 will efficiently complete the entry phase of infection in restrictive non-human primate cells. However, a step that appears to be prior to reverse transcription is blocked. Using human cells transfected with rhesus monkey DNA, TRIM5α, a cytoplasmic protein was shown to be the restrictive element. It has been postulated that TRIM5α binds the HIV capsid protein and targets it for ubiquitination and proteosome-mediated degradation. The affinity of TRIM5α from different primate species for the capsids of different primate lentiviruses determines the patterns of species specificity.

Defense against foreign DNA: APOBEC3G and HIV Vif

The Vif protein is the product of one of the 'accessory' genes of HIV-1 and is required for HIV-1 to replicate in its major cellular target cells, CD4+ T lymphocytes and macrophages. Human cells express APOGEC3G, a cytodine deaminase that can be incorporated into nascent HIV virions, but only if they lack Vif. When Vif-negative virions enter a newly infected cell, reverse transcription of the viral RNA into DNA begins. APOBEC3G then acts upon the first (minus) DNA strand to deaminate cytodines, converting them into uridines, causing a guanosine to adenosine transition in the plus DNA strand. This results in hypermutation of the newly synthesized viral DNA and the resulting virions are typically not viable. The Vif protein counters APOBE3G by tethering it to proteins of the ubiquitin pathway, so that it is degraded and is not incorporated into nascent HIV-1 virions (see Figure 4.11). Recent studies also suggest that APOBEC3G may restrict HIV-1 replication by a second mechanism; in resting cells, a form of APOBEC3G may block HIV-1 after entry.

SEQUENCE OF EVENTS IN HIV INFECTION

Transmission, portal of entry and sequential spread of infection

HIV is transmitted by three major routes, via sexual contact (accounting for >90% of infections worldwide), from mother to child, or by blood or blood products. Mucosal secretions and blood contain both cell-free and cell-associated virus. Cell-free virions are infectious as shown by their ability to initiate experimental infections in primates. The relative importance of cell-associated virus as a vehicle for transmission of HIV-1 infection is unclear since most 'natural' inocula contain cell-free virus as well as infected cells. Under special experimental circumstances, cell cultures can be infected with cell-associated but not with cell-free virus, suggesting that, in some instances, infected cells may be responsible for transmission. Epidemiological studies of breast milk indicate that the level of cell-associated HIV-1 (rather than the level of cell-free virus) is the best predictor of the risk of transmission to the breast-feeding infant.

The details of transmission by sexual contact or other mucosal routes are not entirely clear, specifically how the virus breaches the epithelial or mucosal barrier in order to reach susceptible T lymphocytes or macrophages. Epidemiological studies show that the presence of sexually transmitted diseases increases the risk of HIV infection, which may be due both to ulcerations and abrasions and to increased number of inflammatory cells at the site of infection. In non-human primates, infection can also be initiated by atraumatic application of SIV to the vaginal mucosa or the tonsillar surface and there are several ways in which virus could cross intact epithelial barriers. HIV may bind to the processes of dendritic cells that extend into the vaginal lumen. In the tonsil and rectum, transmission occurs via epithelium that overlies mucosa-associated lymphoid tissue (MALT). In these locations, it is thought that virus may transit epithelial or M (microfold) cells to reach underlying permissive mononuclear cells.

Important insights regarding the early target cells for infection have been gained in the SIV model, which likely mimics some aspects of HIV transmission, although it has been calibrated to produce a much higher rate of infection (Figure 14.3). In the monkey experimentally infected by vaginal or tonsillar application of SIV, the virus can be first detected in the submucosal or lymphoid tissues, primarily in 'resting' CD4+ lymphocytes (~90% of infected cells), with a few infected macrophages or dendritic cells. Dendritic cells in the mucosa can bind and concentrate virus on their surface via DC-SIGN and then carry it to draining lymph nodes, where it is transmitted to permissive T cells.

In the SIV model, within a few days after infection, the local lymphoid tissue is heavily infected, with spread first to draining lymph nodes (~1 week) and thence to distant nodes, spleen and circulating mononuclear cells (1–3 weeks). In addition to the regional lymph nodes, there is a large population of T cells in the lamina propria and Peyer's patches of the gastrointestinal tract, which may be heavily involved shortly after infection.

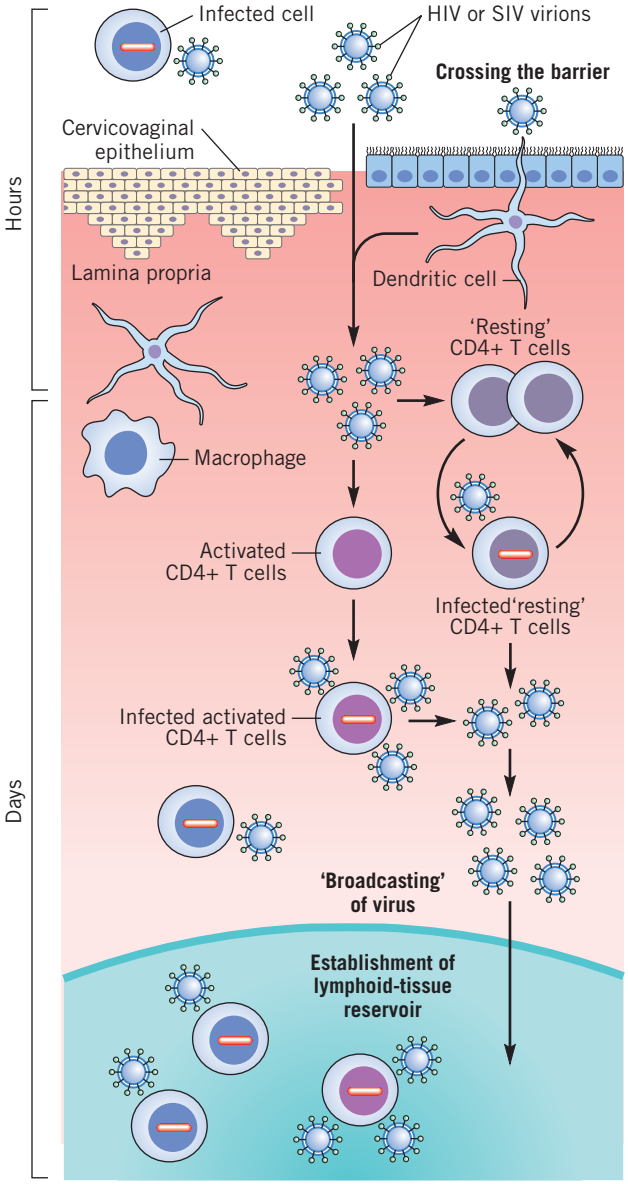

FIGURE 14.3 Cartoon of the early stages in vaginal transmission of SIV to show the role of the major target cells, CD4+ T lymphocytes (resting and activated), dendritic cells and macrophages. After Haase AT. Perils at mucosal front lines for HIV and SIV and their hosts. *Nature Reviews Immunology* 2005, 5: 783–792.

The greatest loss of CD4+ T cells in the body is actually in gastrointestinal-associated lymphoid tissue (GALT), particularly following infection with R5 viruses (Figure 14.4). Within this population, naïve T cells are CCR5− (resistant to HIV or SIV), while memory T cells are CCR5+ (susceptible). Some of the infected CD4+ T lymphocytes are dividing cells, which express high levels of SIV, but the great majority of infected lymphocytes are resting cells that produce less virus and many of them have the memory phenotype. A similar massive depletion of CD4+ lymphocytes in the GALT also occurs early in human infection with HIV-1.

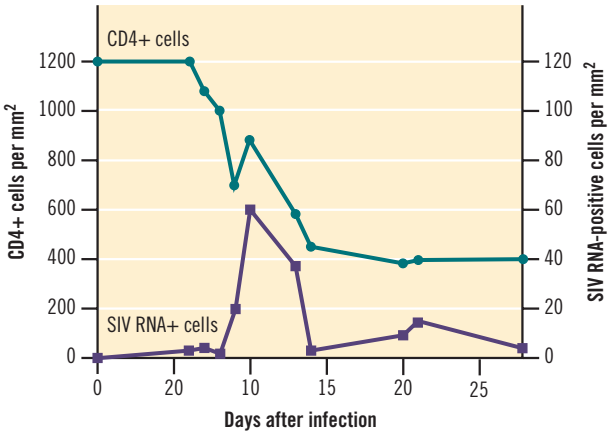

FIGURE 14.4 Virus replication and depletion of CD4+ lymphocytes in the GALT of monkeys during acute SIV infection. Rhesus macaques were infected with an R5 SIV strain (mac251) and infection was followed in the colonic lamina propria (submucosal layer). There was a drastic loss of CD4+ lymphocytes associated with an acute round of SIV replication in CD4+ T cells, most of which were 'resting' since they were negative for surface markers of proliferation or activation (such as Ki67). After Li Q, Duan L, Estes JD *et al.* Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* 2005, 434: 1148–1152, with permission.

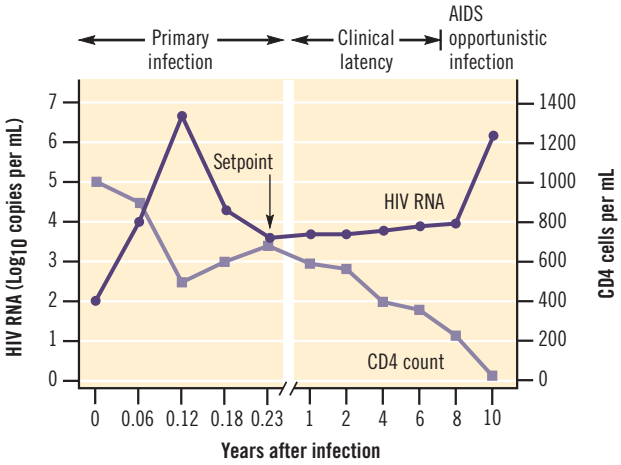

FIGURE 14.5 Typical course of HIV infection as reflected in plasma viremia and concentration of CD4+ lymphocytes in the blood. During primary infection there is widespread dissemination of virus to lymphoid tissues with or without an acute AIDS syndrome. During the acute stage of the infection (4–8 weeks) viremia rises to a high peak and declines; by 3–6 months viremia reaches a plateau, which is often called the 'setpoint' and varies widely in different patients. The onset of AIDS is signaled by a drop in CD4 counts, a rise in viremia level, together with constitutional symptoms associated with opportunistic infections or neoplasms. After Fauci AS, Desrosiers RC. Pathogenesis of HIV and SIV, in Coffin JM, Hughes SH, Varmus HE (eds), *Retroviruses*, Cold spring Harbor Laboratory Press, 1997, with permission.

CD4+ T cells count per μ ml	Stage of infection	Plasma HIV TCID per ml (median and range)	% CD4+ T cells infected (median and range)
>500	II	114 (1–500)	2.7 (0.2–11)
300–499	III	205 (1–500)	21 (2–35)
200–299		381 (25–500)	
<200	IV	1466 (25–5000)	30 (2–65)

TABLE 14.2 HIV produces both a plasma and a cell-associated viremia, that varies in intensity according to stage of infection
TCID: titer of plasma virus expressed as tissue culture infectious doses, based on the highest dilution of plasma that causes a cytopathic effect in the indicator cell culture. The % CD4 + T cells infected: based on in situ PCR. CDC infection stage: II, asymptomatic; III, persistent generalized lymphadenopathy; IV, clinical AIDS. After Bagasra O, Seshamma T, Oakes JW, Pomerantz RJ. High percentages of CD4-positive lymphocytes harbor the HIV-1 provirus in the blood of certain infected individuals. *AIDS* 1993, 7: 1419–1425; Pan L-Z, Werner A, Levy JA. Detection of plasma viremia in individuals at all clinical stages. *Journal of Clinical Microbiology* 1993, 31: 283–288, with permission.

Viremia, CD4 counts and incubation period

HIV produces a viremia that persists throughout the life of the infected individual and can be used to monitor the course of infection. In the blood, HIV is present both in association with infected cells (mainly CD4+ lymphocytes) and as free infectious virus in the plasma (Table 14.2). The level of viremia provides a window on the dynamics of infection. Another useful surrogate for the course of disease is the concentration of CD4+ lymphocytes in the blood, which is inversely related to virus titer and is a harbinger of the functional loss of immune responses during clinical AIDS. Although plasma viral RNA and blood CD4+ lymphocyte levels are generally used to follow the course of HIV-1 infection in patients, they may fail to reflect critical aspects of pathogenesis, such as the concentrations of CD4+ cells in central and peripheral lymphoid tissues and the function of CD8+ effector cells (discussed below).

In the absence of antiretroviral therapy, there is an acute phase of infection (about 2 months duration) with high titer viremia, followed by a subclinical phase with modest levels of viremia, that lasts from 1–>20 years, followed by a phase of clinical AIDS that lasts 1–4 years before death (Figure 14.5). During acute infection, 3–6 weeks after transmission, a mononucleosis-like syndrome occurs in 50–75% of patients, accompanied by a peak in viremia and an acute drop in the CD4+ cell count in the blood. This is followed by induction of an immune response, at 1–3 months, which dampens the infection and is associated with a dramatic drop in blood virus concentration of 10- to 1000-fold below the acute peak level. However, the infection is never completely cleared and viremia usually stabilizes 4–6 months after infection at a level often called the virus 'setpoint'.

During the subclinical phase of infection, virus replication is occurring at a high rate, with concomitant rapid destruction and replacement of CD4+ lymphocytes (described below). The outcome of this dynamic process determines the next steps in infection and explains the diversity in the duration of clinical latency. Rapid progressors develop AIDS in 1–3 years, while long-term non-progressors remain well for 15 years or longer. Outcome is closely related to the virus setpoint (Figure 14.6). In a cohort of infected patients, 90% of the quartile with the highest setpoints progress to AIDS in 5 years, while <10% of the quartile with the lowest setpoint has developed AIDS in that time. Patients with the slowest progression are often dubbed long-term non-progressors

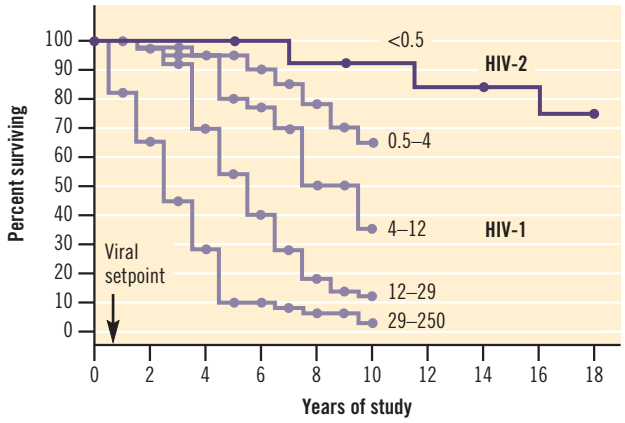

FIGURE 14.6 Progression to AIDS is associated with viremia setpoint. In this example, patients were divided into four quartiles, according to the setpoint determined 6–9 months after infection. At 10 years after infection, >95% of the quartile with the highest setpoints had progressed to AIDS, while <40% of those in the lowest quartile had AIDS. For comparison, data are shown for HIV-2 which is less virulent than HIV-1 and has an even lower frequency of progression to AIDS. Setpoints are shown as RNA copies (×1000) per ml plasma. After Mellors JT, Rinaldo CR Jr, Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science* 1996, 272: 1167–1170; Whittle HC, Ariyoshi K, Rowland-Jones S. HIV-2 and T cell recognition. *Current Opinion in Immunology* 1998, 10: 382–387, with permission.

(LTNPs), generally defined as subjects who are AIDS-free 10 years after infection. Of an initial cohort of infected persons, about 10% became LTNP, but at 20 years after infection only <2% of the original cohort were AIDS-free LTNPs. The slowest progression is seen in patients infected with HIV-2, >50% of whom remain AIDS-free throughout their lives. The ability of the host to contain an HIV infection indefinitely is central to understanding the dynamics of infection and is discussed further below.

Opportunistic infections and neoplasms

The drop in CD4+ lymphocyte levels (normally >1000 cells per μl blood) below a critical threshold (200–300 cells), often accompanied by a rise in virus setpoint, signals the advent of AIDS-defining illnesses. Constitutional symptoms include fever, fatigue, malaise, lymphadenopathy, gastrointestinal symptoms such as diarrhea, weight loss and early evidence of immunodeficiency such as oral candidiasis (caused by *Candida albicans*) and hairy leukoplakia of the tongue (caused by Epstein-Barr virus (EBV) infection of epithelial cells).

Opportunistic infections are caused by a wide spectrum of parasites, including protozoa (such as *Toxoplasma gondii*), fungi (such as such as *C. albicans* and *Pneumocystis carinii*), bacteria (such as *Mycobacterium avium complex* and *M. tuberculosis*) and viruses (such as cytomegalovirus, herpes simplex and varicella zoster). In healthy persons, these agents produce clinically occult infections that rarely manifest as overt illness. Many of these organisms are intracellular parasites that are controlled mainly by the cellular immune response rather than by antibodies and their emergence reflects a decline in cellular immune function as HIV disease progresses. The spectrum of AIDS-associated opportunistic infections differs geographically, reflecting the relative prevalence of different agents. Thus, tuberculosis is much more important as a manifestation of AIDS in developing than in industrialized countries.

Relative to the general population, AIDS patients are at increased risk for a selected number of neoplasms. Among these are polyclonal B cell lymphomas, such as Burkitt's lymphoma (caused by EBV), cervical carcinoma (associated with human papilloma virus (HPV)) and Kaposi's sarcoma (associated with HHV8) (see Chapter 12). It is not clear why specific neoplasms are particularly associated with AIDS, but it probably reflects the compromise of certain immune surveillance mechanisms that are particularly important for control of these cancers, rather than any direct effect of HIV.

IMMUNE RESPONSE TO HIV

HIV proteins as immunogens

HIV proteins are as immunogenic as the comparable proteins of other viruses, if purified and used as experimental immunogens. Some HIV proteins, for instance, gag, are synthesized in much higher copy numbers than others and therefore elicit more robust antibody responses. The intensity of the immune response, whether antibody or CTL, does not predict its ability to prevent de novo infection or to control an ongoing infection. Both of these outcomes depend not only on the potency of the response, but also on whether the targeted epitope is still present in the virus, which is notoriously mutable.

Antibody responses

Most patients develop detectable antibodies against HIV-1 within two months of infection, with highest reactivity against the gag, particularly p24, and envelope (gp120, gp41, or gp160) proteins, usually determined by ELISA (enzyme-linked immunosorbent assay) or Western blot methods. Compared to other viruses, the neutralizing response in the infected but immunocompetent host is typically low when measured against autologous virus. Early neutralizing antibody responses are often narrow (minimal when tested against antigenically unrelated strains of HIV-1), but frequently broaden gradually over several years. Weak neutralizing responses are likely due to the structure of the envelope protein. As reconstructed from X-ray crystallography, the face of the SU protein that binds the CD4 receptor is heavily glycosylated (~24 N linked sites per SU molecule) and the fusion intermediates (see Figure 14.2) that bind to the coreceptors are transient structures. Both these phenomena reduce the probability of inducing epitope-relevant antibodies to block virus entry. In spite of considerable effort, only a few human broadly neutralizing monoclonal antibodies have been identified. Based on their target epitopes, these antibodies fall into four groups: the CD4-binding site on gp120, glycosylation sites on gp120, the CD4-induced site on gp120 and the base of the gp41 molecule just distal to the transmembrane domain.

Neutralizing antibody, when it is present at high levels, is capable of providing significant protection against

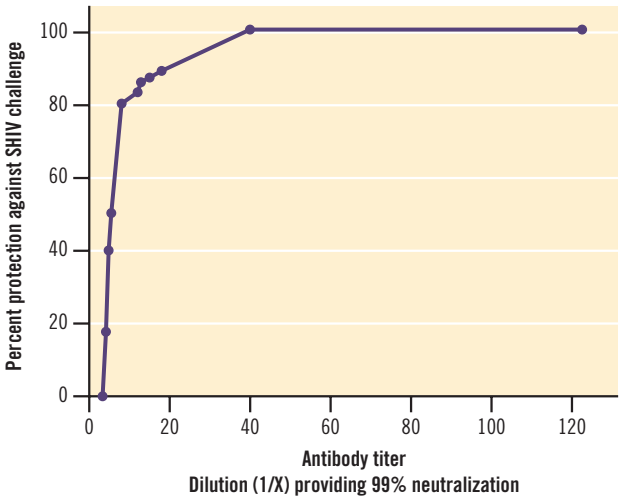

FIGURE 14.7 HIV neutralizing antibodies will protect against a subsequent challenge with SHIV, showing that, once produced, they are as effective as similar antibodies against other viruses. Monkeys were given intravenous injections of immunoglobulin that did or did not contain antibodies against HIV and were challenged intravenously with a pathogenic dual-tropic SHIV DH12, one day later. The neutralizing titer in the plasma of passively immunized monkeys was determined just before challenge. It was calculated that a passive antibody level of ~1:40 protected ~100% of animals and a titer of ~1:7 protected ~50%. Antibody titers are expressed as the highest dilution of plasma that neutralized 100% of 100 TCID (tissue culture infectious doses) of the same virus used for infection. After Nishimura Y, Igarashi T, Haigwood N *et al.* Determination of a statistically valid neutralization titer in plasma that confers protection against Simian-Human immunodeficiency virus challenge following passive transfer of high-titered neutralizing antibodies. *Journal of Virology* 2002, 76: 2123–2130, with permission.

experimental challenge with SIV. Thus, as shown in Figure 14.7, passively acquired neutralizing antibody can protect monkeys against subsequent challenge with a pathogenic SIV or SHIV (a chimeric virus with the HIV envelope on an SIV genetic background). (The protective titers of 1:40 shown in Figure 14.7 are probably much greater than is seen in most HIV-1-infected humans.) Difficulty in inducing an effective neutralizing response probably plays a role in the failure of patients to contain the virus more effectively and has impeded the formulation of an effective pre-exposure vaccine (see Chapter 17).

Cellular immune responses

Cell-mediated immunity to HIV can be assayed by several methods (see Chapter 6) and the technology is under further development and refinement. The most sophisticated methods utilize fluorescent activated cell sorting (FACS) but this requires expensive instrumentation and a highly trained technical staff. Alternatively, an ELISPOT assay can be used to enumerate cells secreting an indicator cytokine, a less complex technology. T lymphocytes prepared from peripheral blood are stimulated with overlapping oligopeptides based on HIV protein sequences and the intracellular synthesis of cytokines (IFNγ or TNFα) is determined. Both FACS and ELISPOT methods can be used to measure responses of CD4+ and CD8+ lymphocytes and have, for the most part, replaced tetramer and cytolytic assays, which examine T cell function more directly.

HIV infected patients raise HIV-specific CD8+ T cell responses against HIV-1 that appear in 1–4 months after infection, concomitant with the decrease in peak viremia. Following acute infection, virus-specific CD8+ cells in the blood usually fall to a low level and exhibit a memory phenotype. HIV-specific CD4+ lymphocytes are rare throughout the course of HIV infection, presumably because they are prime targets for HIV infection and killing. Similar qualitative results have been observed in the SIV model where the time, route, dose and strain of virus can be controlled (Figure 14.8), although the tempo of infection is accelerated relative to HIV-1. In this model, CD8+ effector cells appear about 2 weeks after infection, rise to a high peak at about 4 weeks and decline to a low level at about 6 weeks, following the pattern for many acute viral infections. Concomitant with the rise of effector T cells, there is a drop in plasma virus levels to a setpoint.

What epitopes in HIV-1 are immunogenic? In an exhaustive ELISPOT study, individual HIV-infected patients responded (on average) to ~20 peptides (5% of ~400 peptides spanning the whole HIV open reading frame). Responses among a large group of subjects, were detected to ~85% of all peptides, but the proportion of the population responding to individual peptides varied from 0 to 50%. About 30 of 400 peptides elicited responses in >15% of the population and these 'immunodominant' peptides were most frequently found in Nef and p24 proteins. 'Immunodominant' peptides showed some tendency to have conserved sequences (among HIV isolates) and also showed a tendency to possess amino acid sequences that made them good candidates for C-terminal cleavage by proteosomal enzymes. The HLA segment of human MHC includes HLA-A, HLA-B, and HLA-C gene products, each of which can bind and present peptide epitopes. Among these three loci, CD8+ responses are most frequently directed against epitopes that are HLA-B restricted.

Virus-specific CTLs contribute to the drop in viremia at the end of the acute infection and play an important role in containing viremia levels during persistent infection. Thus, in experimental infection of monkeys with SIV, depletion of CD8 cells with an anti-CD8 antibody reduces the CD8 cell concentration in the blood and is accompanied by a significant transient increase in viremia levels (Figure 14.9).

Reinfection with HIV

Most acute viral infections induce an immune response that provides lifelong protection against a second disease attack. On re-exposure, immune subjects frequently undergo a second infection, but this is much milder than the first infection and is often asymptomatic. Does one infection with HIV protect against a second infection, or against the pathological consequences of the second infection?

For HIV, these questions are difficult to study, because a second infection is hard to detect in the face of the first virus, especially because the first virus may itself

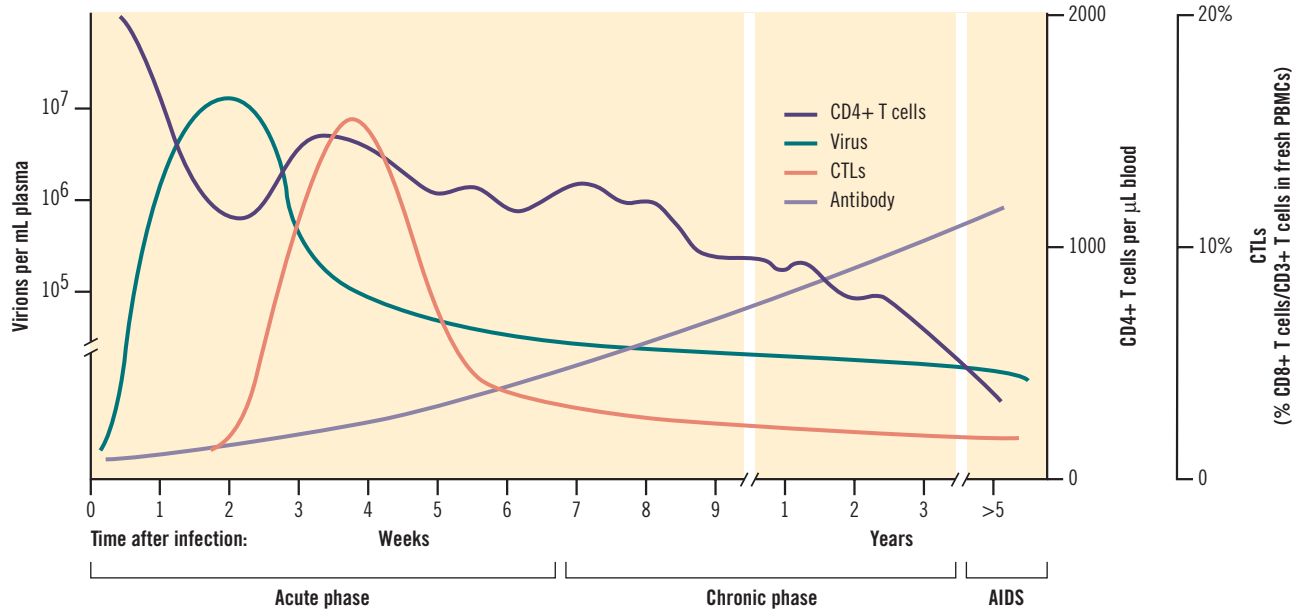

FIGURE 14.8 Average immune response of rhesus macaques following infection with SIV, where virus strain, dose, route and time of infection can be controlled. In this example, SIV infection progresses relatively slowly and the pattern of responses appears to be similar to intermediate speed HIV infection of humans. Virus-specific CD8+ effector T cells were measured in an ELISPOT assay and are shown as a per cent of total T cells (CD3+ cells). Anti-SIV IgG antibody was measured in an ELISA but the titer of neutralizing antibody (not shown) would be minimal. After Goulder PJR, Watkins DI. HIV and SIV CTL escape: implication for vaccine design. *Nature Reviews Immunology* 2004, 4: 630–640, with permission.

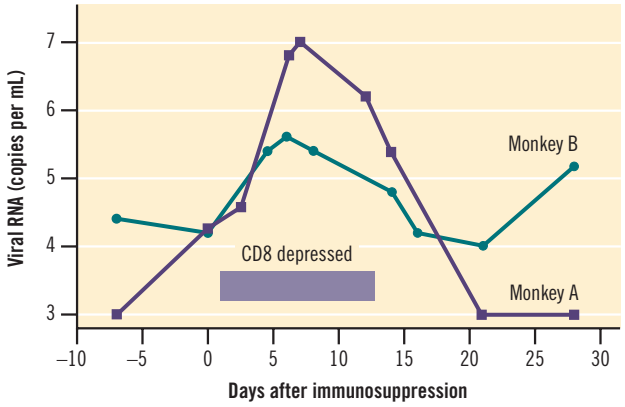

FIGURE 14.9 The cellular immune response plays an important role in the control of SIV infection. Monkeys infected with dual-tropic virulent SIVmac for >9 months had established stable virus setpoints. They were then treated with a potent antibody against CD8, which reduced the level of CD8 T lymphocytes in the blood by >99%. Data on two animals demonstrate the rise in viremia level during the period of immunosuppression and the reconstitution of immune control when immunosuppressive treatment was terminated. The effect of treatment is more pronounced in monkey A with initially lower viremia. After Schmitz JE, Kuroda MJ, Santra S *et al.* Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 1999, 283: 857–860, with permission.

evolve. There have been multiple case reports of HIV re-infection (often called 'superinfection') as well as several studies of infected high-risk subjects. These studies suggest that superinfection may occur at a frequency similar to that of primary infection. When a second virus was identified, the setpoint (level of viremia) was similar to that seen in singly-infected patients for some cases, but a higher viral burden was reported in other cases. In superinfected patients, there was no evident impairment in the cellular immune responses to the initially infecting virus (where this was examined). There is a suggestion that the risk of superinfection is higher during the first year after a primary infection than at a later time, which could reflect a protective effect of a broad neutralizing antibody response, which takes a long time to develop. This hypothesis remains to be tested in a significant number of patients with adequate followup. It will require further studies to determine whether primary infection with HIV fails to induce significant protection against re-infection, in which case HIV would differ from many human viruses.

DYNAMIC ASPECTS OF INFECTION

Virus turnover

During the long period of clinical latency (see Figure 14.5), HIV viremia remains quite stable, although the setpoint varies in different patients. As described in Chapter 2, virus in the blood is constantly removed and replaced (Figure 14.10). Turnover in the plasma is similar to other viruses, with a half-life ($t_{1/2}$) of 10–30 minutes, based on studies of SIV in monkeys. In the presence of antiviral antibody, the $t_{1/2}$ is shortened by two- to fivefold. If the $t_{1/2}$ in plasma is ~15 minutes, then each day 10^8 to 10^{11} virions are shed into the blood in patients whose viral load ranges from 10^3 to 10^6 RNA copies per ml plasma.

The main source of plasma virus is infected activated CD4+ lymphocytes in blood and lymphoid tissues (Figure 14.10). Infected macrophages make a minor contribution

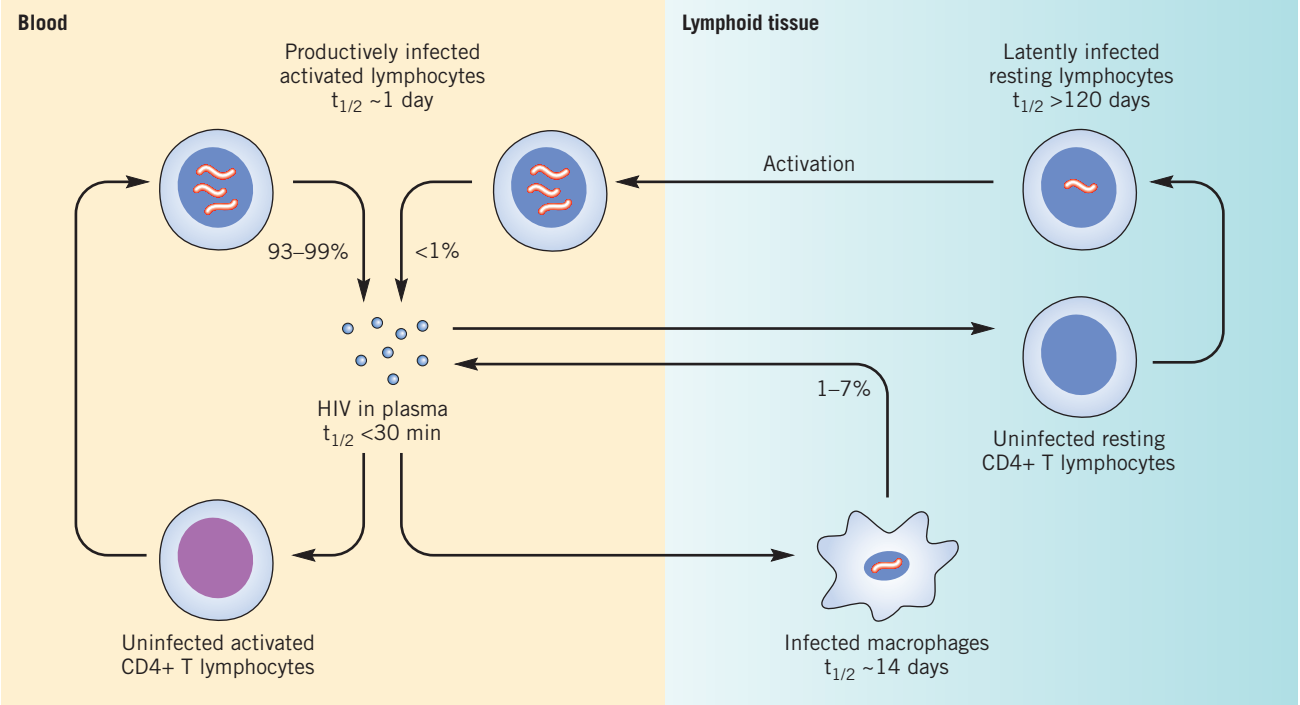

FIGURE 14.10 Kinetics of HIV and SIV in vivo. Plasma virus is produced mainly by infected activated lymphocytes with a modest contribution from infected macrophages. Latent infection of resting lymphocytes is unimportant during active infection but presents an impediment to eradication by HAART (highly active antiretroviral therapy). $t_{1/2}$: half-life. After Igarashi T, Brown C, Azadega A *et al.* Human immunodeficiency virus type 1 neutralizing antibodies accelerate clearance of cell-free virions from blood plasma. *Nature Medicine* 1999, 5: 211–216; Mittler JE, Markowitz M, Ho DD, Perelson AS. Improved estimates for HIV-1 clearance rate and intracellular delay. *AIDS* 1999, 13: 1415–1417; Ramratnam B, Mittler JE, Zhang L *et al.* The decay of the latent reservoir of replication competent HIV-1 is inversely correlated with the extent of residual viral replication during prolonged anti-retroviral therapy. *Nature Medicine* 2000, 6: 82–86; Sharkey ME, Teo I, Greenough T *et al.* Persistence of episomal HIV-1 infection intermediates in patients on highly active anti-retroviral therapy. *Nature Medicine* 2000, 6: 76–81; Zhang L, Dailey PJ, He T *et al.* Rapid clearance of simian immunodeficiency virus particles from plasma of rhesus macaques. *Journal of Virology* 1999, 73: 855–860, with permission.

because of their low levels of virus production. Each infected cell type has a characteristic half-life that can be estimated by following the effects of HAART (highly active antiretroviral therapy) when administered to patients with long-term infection and stable CD4+ lymphocyte levels (Figure 14.11). After commencing HAART, there is a dramatic reduction in plasma viremia, that can be divided into three phases: a rapid drop of ~100-fold over the first 10 days, due to the interruption of most cell-to-cell spread of virus and the die off of infected activated T cells; a slower decrease of ~10-fold over 2 months, reflecting the death of cells with a longer life, probably mainly macrophages; and a plateau that may be below the level of detection but reflects the indefinite persistence of residual latent virus.

HIV persistence

Even after years of treatment with HAART, HIV-1 persists. Evidence for the persistence of a low level of active replication is:

1. the dramatic re-appearance of viremia if HAART is terminated
2. so-called 'blips' (transitory appearance of low levels of replicating virus in the blood) in patients who are under long-term HAART
3. mutations in virus sampled at intervals during prolonged HAART, which are seen in some patients
4. the presence of episomal cDNA intermediates that are labile products indicative of active viral replication.

It is estimated that, after prolonged HAART has reduced residual virus to a plateau (Figure 14.11), there remain 10^3 to 10^7 latently infected cells in individual patients. This reservoir of latently infected cells decays at a half-life estimated at ~6 months or longer, consistent with the lifespan of resting CD4+ memory lymphocytes.

Viral persistence has two main causes, latent infection and active replication. Latently infected resting T cells that carry either a provirus or a pre-integration viral complex cannot be detected and eliminated by antiviral CTLs and yet may be activated intermittently to produce new virus. In addition, in some patients active replication continues at a low level in both resting T lymphocytes and monocytes. HAART intensification, involving the use of five or more drugs simultaneously, accelerated the rate of the drop in viremia but did not eliminate ongoing virus replication. Potentially, the failure of antiretroviral drugs to spread in effective concentration to every cell in solid tissues could play a role and organs such as the thymus, brain, lung and kidney, require further study as possible reservoirs of latent virus.

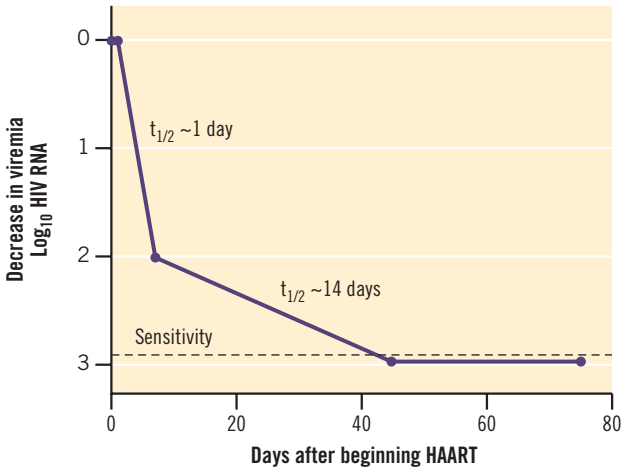

FIGURE 14.11 The reduction in viremia following initiation of HAART (three drugs). The curve can be divided into three phases: early rapid decrease of ~100-fold due to disappearance of infected activated CD4+ lymphocytes; a slower phase due to disappearance of longer lived infected macrophages; and a plateau of persistent infection that may be below the level of detection. After Blankson JN, Finzi D, Pierson TC *et al.* Biphasic decay of latently infected CD4+ cells in acute human immunodeficiency virus type 1 infection. *Journal of Infectious Diseases* 2000, 182: 1636–1642; Grossman Z, Polis M, Feinberg MB *et al.* Ongoing HIV dissemination during HAART. *Nature Medicine* 1999, 5: 1099–1104; Mittler JE, Markowitz M, Ho DD, Perelson AS. Improved estimates for HIV-1 clearance rate and intracellular delay. *AIDS* 1999, 13: 1415–1417; Ramratnam B, Mittler JE, Zhang L *et al.* The decay of the latent reservoir of replication competent HIV-1 is inversely correlated with the extent of residual viral replication during prolonged anti-retroviral therapy. *Nature Medicine* 2000, 6: 82–86; Sharkey ME, Teo I, Greenough T *et al.* Persistence of episomal HIV-1 infection intermediates in patients on highly active anti-retroviral therapy. *Nature Medicine* 2000, 6: 76–81, with permission.

Cellular turnover

In uninfected individuals with stable CD4+ lymphocyte levels in the blood, there is a dynamic equilibrium between the death (or removal) of cells and their replacement. This is altered in HIV-infected persons, resulting in a gradual reduction in CD4+ lymphocyte concentration. Methods have been developed to measure the effect of HIV infection on cellular turnover, using labeling with either BrdU (in monkeys) or tritium-labeled glucose in humans. The results can be expressed as either the half-life ($t_{1/2}$) of circulating CD4+ lymphocytes or the daily replacement of cells as a percent of the total circulating population.

In uninfected healthy subjects, with a CD4+ lymphocyte level of >1000 cells per μl, ~1% of the circulating CD4+ lymphocyte population is replaced daily and the $t_{1/2}$ is ~75 days. In HIV-infected persons, with moderately reduced but stable CD4 counts (~350 cells per μl), the daily replacement rises to ~3% and the $t_{1/2}$ is reduced to ~25 days. The reduced life of CD4+ lymphocytes is due to direct cell killing by HIV, to indirect killing mediated by HIV-specific CTLs and to activation and apoptosis of uninfected CD4+ T cells. The turnover data indicate that HIV-induced reduction in CD4+ lymphocyte half-life is compensated by an increase in the appearance of new CD4+ lymphocytes in the blood. The eventual reduction in CD4 count must reflect a failure of production completely to compensate for cell destruction, but this is a very subtle effect that is too small to measure. In other words, if 99.9% of CD4+ lymphocytes are replaced daily (rather than the 100% in uninfected individuals), the CD4 level would decrease by 3-fold (from ~1000 to ~350) over a period of three years.

Reduced ability to regenerate naïve CD4+ lymphocytes also contributes slightly to the eventual collapse of the immune system. Also, HIV infection targets the thymus and probably perturbs the intrathymic maturation of T cells. When T cells exported from the bone marrow arrive in the thymus they develop into progenitor cells that express CD4 (and other) markers, prior to differentiation into mature T lymphocytes; such progenitor cells are targets for HIV infection. The production of naïve T cells by the thymus is indirectly reflected in the frequency of circulating T lymphocytes that are TREC positive (T cell receptor excision circles, a marker of recent thymic TCR rearrangement) and this proportion is reduced in HIV-infected patients even those whose CD4+ lymphocyte counts are >500 per μl. However, studies in thymectomized macaques indicate that thymic failure does not play a major role in depletion of the CD4+ T cell pool. The turnover of CD4+ lymphocytes in HIV-infected persons who have been successfully treated with HAART gradually returns to that seen in uninfected subjects, with a reduction in daily replacement and an increase in half-life, within about one year after therapy.

CD8 levels in the circulation are usually slightly increased during the subclinical phase of infection but may drop during the end stages of clinical AIDS. However, the turnover of CD8 lymphocytes or of B lymphocytes is increased from the onset of HIV infection. This reflects the HIV-induced generalized activation of all populations of lymphocytes, which reduces cellular half-life, since many activated cells undergo rapid apoptosis. In addition, generalized activation can lead to immune dysregulation and dysfunction of CD8 T cells (discussed below).

VIRAL VARIATION

Retroviruses exhibit a high rate of mutation due to the absence of proofreading during reverse transcription of RNA to DNA. The HIV genome is 10^4 bases long and there is approximately 1 base mismatch per 10^4 to 10^5 nucleotides, or up to one mutation per virion replication. In an HIV-infected subject, ~10^{10} new virions are produced daily, so that each base in the genome undergoes mutation many times each day. Thus, although HIV infection is often initiated by only a few virus particles, the original viral genome quickly evolves into a quasispecies or swarm of genetically related viruses. During in vivo infection, the high mutation rate permits the selection of viral variants that have a selective advantage due to their replication potential or ability to escape host defenses. It has also led to the emergence of viral variants resistant to antiviral drugs.

Biological phenotypes and their evolution during persistent infection

Viruses isolated early in infection have different properties than those present in late infection. Differences include the extent of glycosylation, replication fitness and cytopathic properties. It is unclear whether the change in phenotype causes the development of immunodeficiency or whether immunodeficiency selects for viruses with the late phenotype and both mechanisms may be operative.

The majority of viruses obtained early in HIV-1 infection are R5 (macrophage-tropic viruses that use the CCR5 coreceptor). The importance of R5 viruses in transmission is underlined by the finding that individuals who are homozygous for a genetic deletion of CCR5 are very resistant to infection (discussed below and in Chapter 13). The preferential transmission of R5 viruses is probably due in great part to the expression of R5 on resting T cells (which is thought to be the initial cellular target in the submucosa). Although the level of CCR5 is low it is apparently sufficient to support HIV entry, while CXCR4 expression on these cells is minimal or absent. Another factor may be the differential ability of epithelial or dendritic cells passively to capture and transfer R5 viruses more effectively than X4 viruses.

SIV infection provides further information on the relationship between biological phenotype and the pathogenesis of AIDS. If a cloned SIV is used to infect macaques, isolates obtained during the progression to AIDS show increasing virulence when transmitted to a naïve animal (Figure 14.12). SHIVs (simian human immunodeficiency viruses with an HIV envelope on an SIV genetic backbone) have been used to compare the effect of tropism on pathogenesis. An X4 SHIV spreads to lymph nodes with marked destruction of T cells and a reduction in circulating CD4+ lymphocytes, while an R5 SHIV infects and depletes the GALT (gastrointestinal associated lymphoid tissue) but does not cause a marked reduction in circulating CD4+ lymphocyte count (Figure 14.13).

Immunological escape mutants

HIV infections persist in the presence of an active humoral and cellular response. Because HIV replication inevitably produces large numbers of viral variants, it is not surprising that immunological escape mutants are constantly selected during the course of infection. Since CD8-mediated CTL activity plays a crucial role in the control of HIV infection (see Figure 14.9), viral mutations in immunodominant CTL epitopes have a selective advantage. In HIV infections, mutations accumulate in epitopes to which patients have developed CTL and neutralizing antibody responses, demonstrating that immunological escape plays a role in persistence (Table 14.3).

Non-pathogenic HIV and SIV infections

Untreated HIV-1 infections have a very high fatality rate, close to 100%, which is unusual among viruses of animals

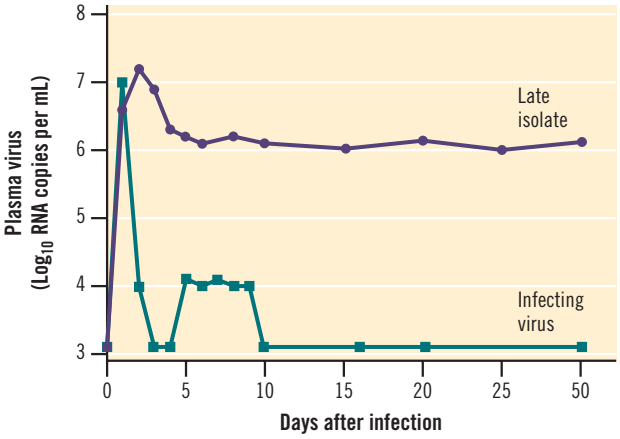

FIGURE 14.12 The virulence of SIV can increase during the course of long-term infection. In this example, macaques (*Macaca nemestrina*) were infected with a cloned macrophage-tropic strain of SIV (Mne clone 8) and virus was isolated at intervals during infection. This figure compares the inoculated virus and a representative late isolate that were used to infect a new group of macaques (one animal per group is shown) and indicates that the late isolate was more virulent than the original clone. After Kimata JT, Kuller L, Anderson DB, Dailey P, Overbaugh J. Emerging cytopathic and antigenic simian immunodeficiency virus variants influence AIDS progression. *Nature Medicine* 1999, 5: 535–541, with permission.

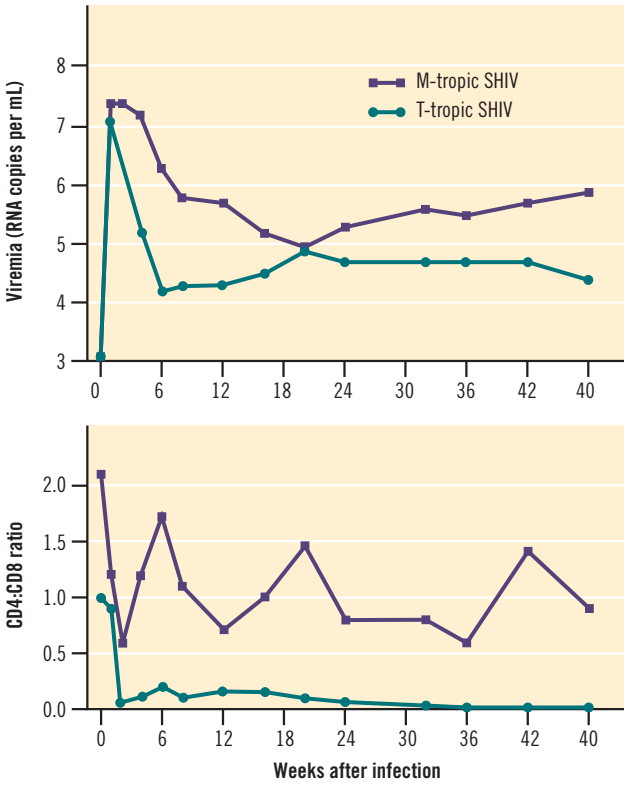

FIGURE 14.13 Macrophage-tropic and T lymphocyte-tropic viruses differ in their pathogenesis. This figure compares X4 (T-tropic) and R5 (M-tropic) strains of SHIV (simian human immunodeficiency virus). Upper panel: both viruses produced similar levels of viremia. Lower panel: X4 SHIV reduced circulating CD4+ T lymphocytes, while R5 SHIV did not reduce. The X4 virus infected mainly lymph nodes while the R5 virus depleted GALT (gastrointestinal-associated lymphoid tissue). After Harouse JM, Gettle A, Tan RCH, Blanchard J, Cheng-Mayer C. Distinct pathogenic sequelae in rhesus macaques infected with CCR5 or CXCR4-tropic strains of SHIV. *Science*, 1999, 284: 816–881, with permission.

or humans. However, lower virulence is seen with HIV-2 in humans while HIV-1 in chimpanzees (the original host) rarely causes illness or death. Many SIVs have been isolated from different species of monkeys and most of them appear to be relatively benign in their natural hosts, such as SIVagm in African green monkeys or SIVsm in sooty mangabeys. Pathogenic SIV infections have mainly resulted from passage into a monkey species (Asian-origin monkeys) other than the natural host (African-origin monkeys). High virulence, as exhibited by HIV-1 in humans, is clearly not required for survival of lymphotropic lentiviruses and may be a phenomenon associated with recent transmission across a species barrier.

Studies of SIV strains in their natural hosts indicate that there are at least two distinct mechanisms that explain the benign nature of non-pathogenic infections. In some instances, virus replication is contained at low levels, similar to those seen in long-term non-progressors infected with HIV-1. Under such circumstances, homeostatic mechanisms are apparently able to replace lost CD4+ lymphocytes, leading to asymptomatic lifelong infections. However, in other examples, such as SIVsm infection of sooty mangabeys, virus replicates to high titers, but CD4+ T cells are not reduced and there is little evidence of generalized activation of lymphocytes. In this instance, the benign nature of the infection is associated with a lack of T cell hyperactivation that is seen in pathogenic SIV infections and in human AIDS.

Clades

Extensive genomic studies have identified nine major subtypes (clades) within the M group of HIV-1 that causes >95% of HIV-1 infections. Clades and recombinants between the clades appear to have arisen in Africa, where HIV-1 first emerged. It is not clear whether there are any consistent biological or epidemiological differences between the clades; some subtle differences have been noted, but all of them spread readily in humans. Outside of the African continent, certain clades predominate (such as clade B in North America), presumably due to a founder effect when the virus was originally introduced. Clades do not correspond to immunotypes and there is considerable cross-antigenicity between clades, based on both neutralization (using the few sera with strong neutralizing capacity) and CTL assays.

MECHANISMS OF IMMUNE SUPPRESSION

Paradox: CD4+ lymphocyte depletion versus CD8+ lymphocyte dysfunction

The appearance of functional immunodeficiency in HIV-infected patients presumably reflects the inability of cellular immune mechanisms, particularly CD8+ T lymphocytes, to contain opportunistic infections. When treatment-naïve patients with opportunistic infections begin therapy with effective HAART, their opportunistic infections 'melt away', implying a reconstitution of effector CD8 lymphocytes.

This presents an apparent paradox, since CD4+ lymphocytes and macrophages, not CD8 cells, are targeted by HIV. The reduction of CD8 function is probably due to the concatenation of several effects:

1. There is a paucity of CD4+ helper cells that are required to induce new antigen-specific CD8+ effector cells from the post-thymus naïve T cell pool. In particular, CD4+ lymphocyte help is required to generate and maintain CD8+ memory cells and this potential defect may contribute to the loss of effector function in chronic infections.
2. The state of general lymphocyte activation produced by HIV infection results in a high level of apoptosis that causes a non-specific physiological exhaustion of

Category of HIV-1 isolate according to sequence of gag 240–249 epitope	Patients with HLA-B57 or HLA-B5801 haplotype (124 patients)	Patients with other haplotypes (187 patients)
	Percent of patients whose virus was classified in the indicated category	
Consensus sequence in gag 240–249 epitope	7	77
Known escape mutation in gag 240–249 epitope (T242X)	78	3
Other mutations within gag 240–249 epitope	15	20
Totals	100	100

TABLE 14.3 HIV-1 escape variants are selected in the presence of an immunodominant epitope. HIV-1 isolates from patients with chronic HIV infections (clades B and C) were sequenced to determine the amino acids at gag 240–249. This 10 amino acid sequence is conserved in each clade and is known to be an immunodominant cellular epitope in patients with HLA haplotypes B57 and B5801. Compared to viruses from patients with other HLA haplotypes, a high proportion of viruses from patients with HLA haplotypes B57 or B5801 showed an escape mutation at position 242, evidence of the strong pressure for selection of variants that would escape the CTL response at this epitope
After Leslie AJ, Pfafferott KJ, Chetty P *et al.* HIV evolution: CTL escape mutation and reversion after transmission. *Nature Medicine* 2004, 10: 282–289, with permission.

committed clones of CD8 cells. Non-pathogenic SIV models, in which high levels of viral infection are well tolerated, strongly imply that generalized immune activation and, perhaps other secondary effects of infection, play a key role in the pathogenesis of immunodeficiency.

3. Continuous antigen-specific immune stimulation, produced by ongoing infections, activates pools of parasite-specific memory cells and eventually exhausts them. Together, these effects erode cellular immune surveillance of opportunistic pathogens.

GENETIC DETERMINANTS OF HOST SUSCEPTIBILITY TO HIV

Epidemiologic studies have identified a number of genetic determinants that influence susceptibility to infection or the rate of progression to AIDS (Table 14.4). These determinants mainly fall into two categories, those that alter coreceptor availability and those in HLA loci (see Table 13.7). The HLA alleles appear to act by determining the selection of HIV peptides for immune presentation. Coreceptor genes determine the level of expression of coreceptors or the expression of coreceptor ligands (such as chemokines) that could reduce receptor availability for HIV attachment. The most prominent mutation is the Δ32 deletion in CCR5 that abrogates or reduces the expression of that gene. In its homozygous form, Δ32 markedly reduces the risk of infection and, in its heterozygous form, reduces the rate of progression to AIDS (see Table 13.6).

REPRISE

HIV is a persistent virus that has a complex pathogenesis with many unusual features. Characteristics that define the infection include:

1. involvement of the helper subset of T lymphocytes and macrophages due to the use of CD4 as the primary cellular receptor
2. lifelong persistence mainly due to the occurrence of latency in resting CD4+ lymphocytes in which an integrated provirus can be maintained for many years, enhanced by the development of variants that evade epitope-specific neutralizing antibodies and CTLs
3. the ability to destroy activated CD4+ lymphocytes at a high rate, which eventually exhausts homeostatic replacement by the host
4. the initiation of an acquired immunodeficiency due to the loss of CD4+ T cells and the perturbation of the function of CD8 effector T lymphocytes.

This unique constellation of characteristics combines to produce an infection that – absent treatment – is close to 100% fatal.

Another unusual characteristic of HIV is its extremely low transmission (risk <1:100 contacts) by sexual contact, the primary mode of host-to-host spread, reflecting the requirement for the transmitted virus to contact T lymphocytes and/or macrophages and the inability to infect epithelial cells. Similarly, a minority of HIV-infected pregnant women transmit HIV to their infants (up to 35% of women who breastfeed). Low transmission rate and 100% fatality would ordinarily limit the ability of a virus to survive in its host population.

Genetic locus	Genetic context	Biological effect	Influence on progression to AIDS
CCR5	Homozygous	Δ32 mutation in CCR5 abrogates or reduces CCR5 expression	Prevents virus infection
	Heterozygous		Retards progression
CCR5	Homozygous	P1 mutation in promoter for CCR5	Accelerates progression
CCR2	64I mutation Heterozygous	Unknown	Retards progression
CX3CR1	1249 mutation Homozygous	Mutation reduces chemokine binding	Accelerates progression
CCL3L1	Low number of gene duplications	Chemokine ligand for CCR5	Accelerates progression
HLA-B*35	Homozygous	Unknown	Accelerates progression
HLA-B*57	Homozygous	Presents peptides with broad HIV representation	Retards progression
HLA-B*27	Homozygous	Presents peptide which is a conserved immunodominant epitope that is under structural constraint	Retards progression
HLA-A, -B, -C	Homozygous	Reduces number of peptides presented (HLA-B mainly)	Accelerates progression

TABLE 14.4 Mutations that influence susceptibility to HIV infection or the rate of progression to AIDS

Paradoxically, HIV has spread rampantly in spite of these limitations, due mainly to the long incubation period that provides many opportunities for infected but apparently healthy persons to transmit infection.

Furthermore, HIV has been peculiarly resistant to control measures. This can be ascribed in part to the social stigma associated with an infection that is transmitted by sexual contact or the use of injected drugs. In addition, HIV often targets poor countries or socially disadvantaged subgroups in industrialized countries. The insidious nature of an infection that is widely seeded before impacting an infected population aggravates the problem. In summary, the features of HIV pathogenesis have combined to cause a global pandemic that presents the greatest infectious disease challenge in the history of medical science.

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PART IV

Ecology and Control of Viral Infections

Chapter 15

Emerging Viral Diseases

Neal Nathanson and Frederick A. Murphy

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REPRISE

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One of the most dramatic aspects of virology is the emergence of new virus diseases, which often receives widespread attention from the scientific community and the lay public. Considering that the discipline of animal virology was established over 100 years ago, it may seem surprising that new virus diseases are still being discovered. How this happens is the subject of this chapter.

HOW NEW VIRAL DISEASES EMERGE

There are many recent books and reviews (see Further Reading) that list the plethora of determinants that can lead to the emergence of infectious diseases (Table 15.1). In this chapter we focus on those determinants that relate to viral pathogenesis (Sidebar15.1) and do not deal with many societal and environmental factors that can be instrumental in disease emergence.

Discovery of the etiology of an existing disease

In some instances, the 'emergence' of a viral disease represents the original identification of the cause of a well recognized disease. An example is La Crosse virus, a mosquito-transmitted bunyavirus that was first isolated from a fatal case of encephalitis in 1964. The isolation of the causal agent and the development of serological tests made it possible to distinguish La Crosse encephalitis from the rubric of 'arbovirus encephalitis, etiology unknown'. Since that time, about 100 cases have been reported annually, without any significant increase since the 1970s. It appears that the emergence of this 'new' disease reflected only the newfound ability to identify this etiologic entity, rather than any true change in its occurrence.

Hantavirus pulmonary syndrome is an example of the 'emergence' of an existing but previously unrecognized disease. In 1993, in the four corners area of the southwestern USA, there occurred a small outbreak of cases of acute pulmonary illness with a high mortality (Figure 15.1). Epidemiologic and laboratory investigation rapidly identified the causal agent, a previously

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Category	Determinant
Human demographics and behavior	Population growth, density and distribution
	Immunosuppression
	Sexual activity and substance abuse
Technology and industry	Modern medicine
	Food processing and handling
	Water treatment
Economic development and land use	Dam building
	Reforestation
	Global warming
International travel and commerce	Travel
	Commerce
Microbial adaptation and change	Natural variation/mutation
	Selective pressure and the development of resistance
	Microbes as cofactors in chronic disease
Breakdown of public health measures	Inadequate sanitation
	Complacency
	War

TABLE 15.1 Some of the factors that lead to emergence or re-emergence of viral diseases
After Lederberg J, Shope RE, Oaks Jr SC (eds). *Emerging infections*, National Academy Press, Washington, DC, 1992, with permission.

SIDEBAR 15.1

How new virus diseases emerge or re-emerge:

- discovery of the etiology of an existing disease
- accumulation of susceptible hosts
- increase in disease caused by an existing virus
- virus new to a specific population

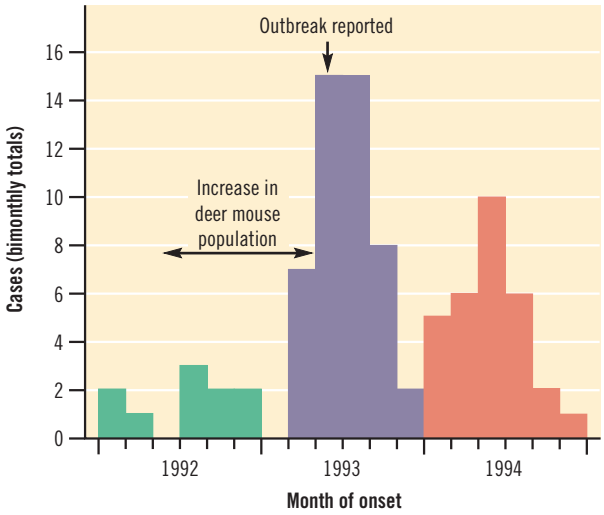

FIGURE 15.1 Cases of hantavirus pulmonary syndrome by month of onset, USA, 1992–1994, omitting 16 cases with onsets prior to 1992. Redrawn from Kahn AS, Khabbaz RF, Armstrong LR *et al*. Hanatvirus pulmonary syndrome: the first 100 cases. *Journal of Infectious Diseases* 1996, 173: 1297–1303, with permission.

unknown hantavirus, now named Sin Nombre virus (SNV). SNV is an indigenous virus of deer mice (*Peromyscus maniculatus*) that are persistently infected and excrete the virus. Apparently, deer mice produce virus-infected aerosols and, when they infest human dwellings, this can result in occasional human infections. The 1993 outbreak is thought to reflect a transient rise in deer mouse populations associated with an unusual crop of pine nuts, a major food source for these rodents. Subsequent surveillance has continued to detect cases of hantavirus pulmonary syndrome. The 'emergence' of SNV represents the recognition of a long-existing agent and disease that first came to attention because of an unusual cluster of cases.

Increase in disease caused by an existing virus

On occasion, a virus that is already widespread in a population can emerge as a cause of epidemic or endemic disease, due to an increase in the ratio of cases to infections. Such an increase can be caused by either an increase in host susceptibility or enhancement of the virulence of the virus. Although counterintuitive, there are some dramatic instances of such phenomena.

Increase in host susceptibility

Poliovirus

Poliomyelitis first appeared as a cause of summer outbreaks of acute infantile paralysis in Sweden and the USA late in the 19th century (Figure 15.2). Isolated cases of infantile paralysis had been recorded in prior centuries and circumstantial evidence indicates that poliomyelitis probably occurred in early recorded history. Why then did poliomyelitis emerge abruptly as an epidemic disease? When personal hygiene and public health were primitive, poliovirus circulated as a readily transmitted enterovirus and most infants were infected while they still carried maternal antibodies (up to 9–12 months of age). Under these circumstances, the virus produced immunizing infections of the enteric tract but circulating antibodies prevented invasion of the spinal cord. With

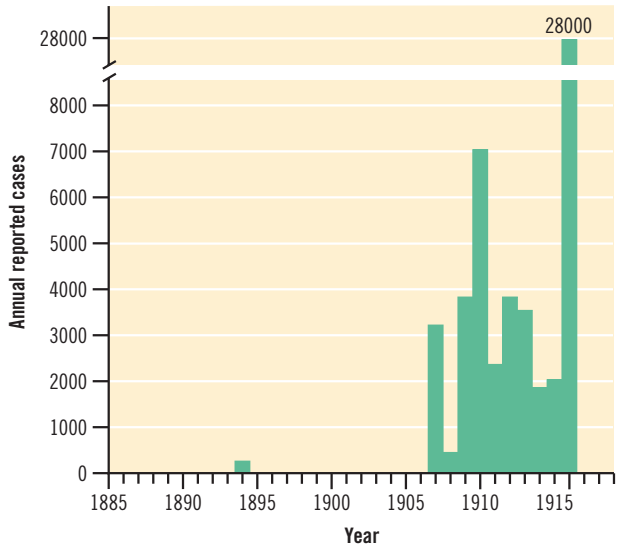

FIGURE 15.2 The appearance of epidemic poliomyelitis in the USA, 1885–1916. The graph is based on reported cases (mainly paralytic) during an era when reporting was estimated at about 50%. Data from Lavinder CH, Freeman SW, Frost WH. *Epidemiologic studies of poliomyelitis in New York City and the northeastern United States during the year 1916*, United States Public Health Service, Washington, 1918, with permission.

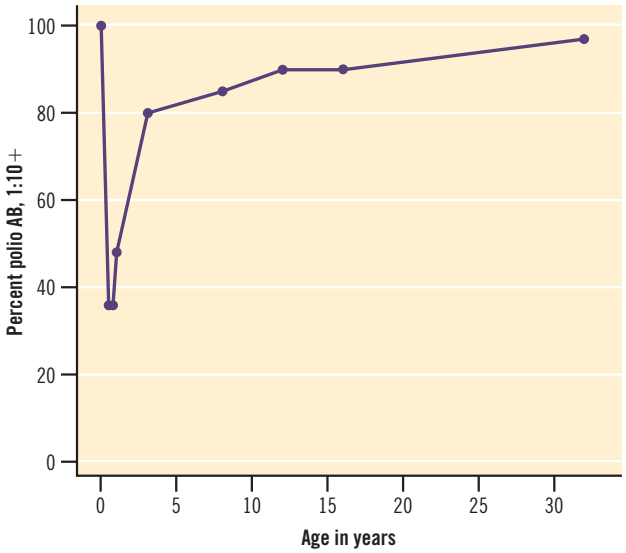

FIGURE 15.3 Age-specific proportion of native Moroccans with type 1 neutralizing antibody at 1:10 titer or greater, Casablanca, 1953. Data from Paul J, Horstmann D. A survey of poliomyelitis virus antibodies in French Morocco. *American Journal of Tropical Medicine and Hygiene* 1955, 4: 512–524, with permission.

the improvement of personal hygiene in the late 19th century, infections were delayed until 1–3 years of age, after the waning of maternal antibodies. Initial infections now occurred in susceptible children, resulting in viremia and spread to the central nervous system. Hence, the dramatic emergence of outbreaks of infantile paralysis. This reconstruction is supported by seroepidemiological studies conducted in the 1950s, prior to the introduction of poliovirus vaccine (Figure 15.3).

Increase in viral virulence

Avian influenza virus

Viruses may undergo sudden increases in virulence resulting in emergence of dramatic outbreaks. The outbreak of lethal avian influenza in Pennsylvania in 1983 is one documented example. In eastern Pennsylvania, avian influenza appeared in chicken farms early in 1983, but the virus was relatively innocuous and most infections were mild. However, in the fall of that year a rapidly fatal influenza pandemic spread rapidly through the same farms. When virus isolates from the Spring and Fall were compared, it appeared that both isolates had almost identical genomes, but there was a single point mutation in the viral hemagglutinin that facilitated the cleavage of the hemagglutinin, an essential step to render nascent virions infectious. The cellular host range of the virus was extended and the virus could now replicate outside the respiratory tract, markedly increasing its virulence (discussed in Chapters 4 and 9). This point mutation led to the emergence of an overwhelming pandemic, which was only controlled by a widespread slaughter program involving millions of birds. Similar outbreaks of avian influenza have occurred subsequently in other countries.

Oral poliovirus vaccine

Oral poliovirus vaccine (OPV) is a trivalent vaccine that includes strains representing types 1, 2 and 3 poliovirus, which are antigenically distinct subtypes. Each vaccine strain was derived from a wild poliovirus isolate and carries up to 10 distinct attenuating mutations. However, on human passage there is a selection for revertant strains that are as virulent as wild polioviruses. On rare occasion, these revertant viruses can cause paralytic poliomyelitis in primary vaccinees, at the frequency of about 1 case per 500 000 primary vaccinees.

It has recently been discovered that revertant viruses can also cause outbreaks of poliomyelitis and several such outbreaks, ranging up to 50 cases each, have been documented. These outbreaks occur under special epidemiological circumstances, where mass immunization campaigns with OPV are only partially successful, so that a large proportion of the population (40–80%) is not immunized. Revertant polioviruses are excreted by vaccinees and can then spread from person to person among unvaccinated susceptible individuals. Such outbreaks are indistinguishable epidemiologically from outbreaks caused by wildtype polioviruses, but sequencing of the virus isolates have shown that they are much more closely related to OPV than to wildtype polioviruses and are therefore called circulating vaccine-derived polioviruses (cVDPV). cVDPV present a problem that complicates the strategy for the 'endgame' in the global campaign to eradicate polioviruses, which is well on its way to completion.

Accumulation of susceptible hosts and viral re-emergence

A virus that is endemic in a population may 'fade out' and disappear, because the number of susceptibles has

fallen below the critical level required for perpetuation in that population. If the population is somewhat isolated, the virus may remain absent for many years. During this interval, there will be an accumulation of birth cohorts of children who are susceptible. If the virus is then re-introduced, it can 're-emerge' as an acute outbreak. In the years 1900 to 1950, Iceland had a population of about 200 000, which was too small to maintain measles virus and measles periodically disappeared. When travelers to Iceland reintroduced the virus, measles re-emerged in epidemic proportions (Figure 15.4).

Virus new to a specific population

West Nile virus

On occasion, a virus can enter and spread in a region where it had never previously circulated, leading to the emergence of a disease new to that locale. A dramatic example is afforded by the emergence of West Nile virus (WNV) in the USA, beginning in 1999. WNV, like most arboviruses, is usually confined to a finite geographic area, based on the range of its vertebrate reservoir hosts and permissive vectors. In an unusual and perhaps unprecedented event, WNV was imported into New York City, probably by the introduction of infected vector mosquitoes that were inadvertent passengers on a flight from the Middle East, where the virus is enzootic. This hypothesis was supported by the finding that the genomic sequence of the New York isolates was closely related to the sequence of contemporary isolates from Israel. Some American mosquito species were competent vectors for WNV and certain avian species, such as American crows, were highly susceptible. As a result, West Nile encephalitis emerged as an important disease new to the USA and, over a period of several years, has spread across the continent, finally reaching the west coast.

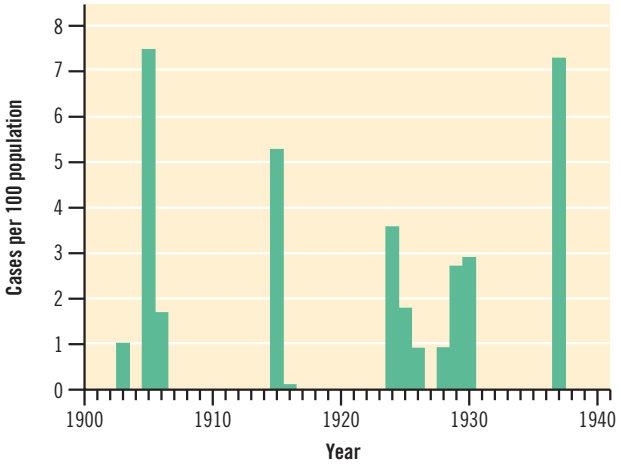

FIGURE 15.4 Reported cases of measles in Iceland, 1900 to 1940. Data from Tauxe R. *Measles incidence in Iceland*, unpublished report, 1979.

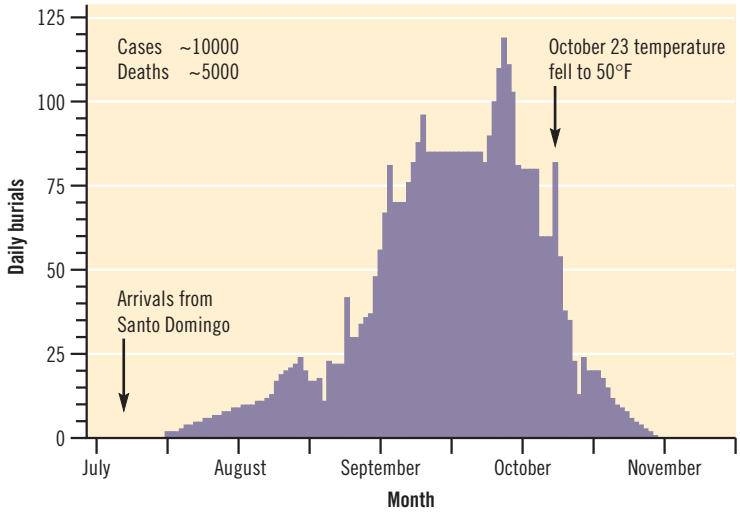

FIGURE 15.5 Yellow fever in Philadelphia in the summer of 1793. This figure was reconstructed from anecdotal accounts of burials as recounted in Powell JM. *Bring out your dead: the epidemic of yellow fever in Philadelphia in 1793*, University of Pennsylvania Press, Philadelphia, 1949, reprinted 1993, with permission.

Yellow fever

In the 18th and 19th centuries, there were periodic outbreaks of yellow fever in port cities of the eastern and southern USA, sites where yellow fever was not endemic but was repeatedly introduced. One notable epidemic occurred in Philadelphia in 1793, when yellow fever virus was imported by sailors and passengers on ships from the Caribbean. In this case, the vector mosquitoes, *Aedes aegypti*, bred in standing water on shipboard and caused cases of yellow fever among the crew and passengers, maintaining the virus through several transmission cycles; once docked, infections spread from the waterfront area through the city. The virus could spread because *Aedes aegypti* mosquitoes were indigenous in the city and most of the human population were highly susceptible to the virus. The resulting epidemic killed 5–10% of the inhabitants of Philadelphia and closed down the federal government for the summer of 1793. President George Washington and Secretary of State Thomas Jefferson were among those who fled the city. With the first frosts in October, the mosquito population was drastically reduced, the transmission cycle was broken, the virus disappeared and the epidemic came to a close (Figure 15.5).

ZOONOTIC INFECTIONS AS A SOURCE OF EMERGING VIRAL DISEASES

An important cause of emerging virus diseases is zoonotic infections, i.e. infections of animals that can be transmitted to humans, either by direct contact, by virus-laden aerosols, or by insect vectors. All zoonotic viruses have one or more animal reservoir hosts, which play an important role in the epidemiological dynamics of human infections. Although many zoonotic viruses can be transmitted to humans on occasion, their relative ability to spread to humans determines whether or not they emerge as significant new virus diseases of mankind (Table 15.2).

Deadend hosts

Most zoonotic viruses that are transmitted to humans cannot be spread directly from person to person, so humans are considered to be 'deadend hosts'. One familiar example is rabies, which is enzootic in several animal hosts, such as foxes, dogs, raccoons and bats. Humans are infected by the bite of a rabid animal (see Figure 1.1) or by aerosol exposure (in caves with roosting bats). Several zoonotic arenaviruses, such as lymphocytic choriomeningitis, Machupo (Bolivian hemorrhagic fever) and Junin (Argentine hemorrhagic fever) viruses, are likely transmitted from the reservoir host (wild rodents) by inhalation of contaminated aerosols.

Arboviruses

There are more than 500 viruses, belonging to several virus families, that are also classified as arboviruses (arthropod-borne viruses), based on a vertebrate–insect maintenance cycle in nature. Arboviruses replicate in both the vertebrate host and the insect vector and transmission occurs when the vector takes a blood meal. Typically, arbovirus have one or several vertebrate hosts and are transmitted by a narrow range of insects, usually one or a few species of mosquitoes or ticks. Humans are not the reservoir vertebrate hosts of any arboviruses, with two exceptions. In urban settings, dengue and urban yellow fever viruses can be maintained by a mosquito–human

Extent of human to human spread *commonness*	Virus	Maintenance cycle in nature
Humans are deadend hosts (representative examples) *common*	West Nile (flavivirus)	Mosquitoes, birds
	Yellow fever (flavivirus)	Mosquitoes, primates
	Eastern equine encephalitis (alphavirus)	Mosquitoes, birds
	La Crosse encephalitis (bunyavirus)	Mosquitoes, wild rodents
	Rabies (rhabdovirus)	Raccoons, skunks, bats
	Nipah (paramyxovirus)	Bats, pigs
Limited (<10) human-to-human transmissions *uncommon*	Crimean Congo hemorrhagic fever (bunyavirus) 1–3 serial infections	Ticks, agricultural and wild animals
	Lassa, Machupo, Junin (arenavirus) 1–8 serial infections	Rodents
	Monkeypox (poxvirus) <6 serial infections	Rodents
	Ebola, Marburg (filovirus) 1–4 serial infections	Unknown
	Swine influenza (influenza virus Type A) ? 1–3 serial infections	Pigs
Unlimited human-to-human transmission (a new human virus) *rare*	SARS, (coronavirus)	Palm civets, other animals?
	Influenza (type A influenza virus)	Birds, pigs
	HIV (lentivirus)	Chimpanzees

TABLE 15.2 Zoonotic virus infections of humans and the extent of their human-to-human transmission

FIGURE 15.6 Global distribution of countries with SARS cases, 2002–2003. Large red circle: point of origin of epidemic; red dots: countries with first cases November 2002 through February 2003; black dots: countries with first cases March through May, 2003. After data in www.who.int/csr/sars/country/table 2004_04_21, 04 11 05.

cycle. Yet humans can be infected by many of these viruses, if they happen to be bitten by an infected insect vector.

In most instances, arbovirus-infected humans are deadend hosts for several reasons. Often, infected humans are not sufficiently permissive to experience a high titer viremia, so they cannot serve as effective links in the transmission cycle. Also, many insect vectors competent to transmit a zoonotic arbovirus prefer non-human hosts as a blood source, reducing the likelihood of transmission from human to vector.

Limited spread among humans

As Table 15.2 shows, a few zoonotic viruses can be transmitted from human to human, at least for a few passages and can emerge as the cause of outbreaks involving a few to several hundred cases. Since many viruses in this group cause a high mortality in humans, even a small outbreak constitutes a public health emergency. These viruses belong to many different virus families and there is no obvious biological clue why they should be able to spread from human to human, in contrast to other closely related viruses. Typically, infections are mainly limited to caregivers or family members who have intimate contact with patients, often in a hospital setting. Most of these viruses are transmitted by the respiratory route and it is inferred that the infected patients produce virus-laden aerosols, either by coughing or from sloughing skin (monkeypox). However, transmission is marginal and requires close contact with patient's bodily fluids, so that most outbreaks end after fewer than 5 to 10 serial transmissions, either spontaneously or due to infection-control practices.

Crossing the species barrier

In the history of modern virology (the last 50 years) there are very few documented instances where zoonotic viruses have established themselves in the human population and have emerged as new viral diseases (see Table 15.2). Most viruses have evolved to optimize their ability to be perpetuated within one or a few host species and this creates what is sometimes called the 'species barrier'. It may be speculated that the species barrier accounts for the infrequency of emergence of new human viral diseases of zoonotic origin. By the same token, it is possible that a virus must undergo some adaptive mutations to become established in a new species.

SARS coronavirus (SARS CoV)

In November, 2002, an outbreak of severe acute respiratory disease began in Guangdong Province, in southeast China near the Hong Kong border. In retrospect, the first cases were concentrated in food handlers, who then spread the virus to the general population in that region. Although not recognized immediately as a new disease, the outbreak continued to spread both locally and in other parts of China. In February, 2003, a physician who was in the incubation period of the infection, traveled to Hong Kong, where he transmitted SARS to a large number of contacts in a hotel. These persons, in turn, spread the infection to Singapore, Taiwan, Vietnam and Canada, initiating a global pandemic that eventually involved almost 30 countries (Figure 15.6, Table 15.3). From patient samples, several research groups isolated a novel coronavirus,

Countries	Cases	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
China	5327										
Hong Kong	1755										
Taiwan	346										
Singapore	238										
Canada	251										
5 Other countries	111										
11 Other countries	50										
6 Other countries	11										
2 Other countries	2										
Total: 29 Countries	8091										

TABLE 15.3 The SARS epidemic of 2002–2003, to show the numbers of cases and the duration of the outbreak (shaded area) by country
Countries with small numbers of cases are grouped according to date of first case. Other countries: Australia, Macao, France, Germany, India, Indonesia, Italy, Kuwait, Malaysia, Mongolia, New Zealand, Philippines, Republic of Ireland, Republic of Korea, Romania, Russian Federation, South Africa, Spain, Sweden, Switzerland, Thailand, UK, USA, Vietnam. After data in www.who.int/csr/sars/country/table 2004_04_21, 04 11 05.

which has been named the SARS CoV. Clearly, this virus is new to the human population and there is circumstantial evidence that it was contracted from infected exotic food animals that are raised for restaurants in Guangdong Province.

SARS CoV went through a large number of human passages (perhaps 25) before being contained by primitive control measures, such as respiratory precautions, isolation and quarantine. As of this writing (2006), the virus has been eliminated from the human population, but the recent global outbreak showed that this virus could be maintained by human-to-human transmission. From that perspective, it is potentially capable of becoming an indigenous virus of humans. Since many coronaviruses infect the respiratory system and are transmitted by aerosol, the SARS virus did not have to undergo any change in its pathogenesis. However, the virus did have to replicate efficiently in cells of the human respiratory tract and it is unknown whether this required some adaptive mutations from the virus that is enzootic in its reservoir hosts. Recent studies suggest that horseshoe bats (genus *Rhinolophus*) are the reservoir hosts and palm civets, consumed as food in China, are intermediary hosts, for SARS CoV.

Type A influenza virus

Genetic evidence strongly implicates avian and porcine type A influenza viruses as the source of some past pandemics of human influenza. It appears that new epidemic strains are derived as reassortants between the hemagglutinin (and the neuraminidase in some cases) of avian influenza viruses with other genes of existing human influenza viruses. The new surface proteins provide a novel antigenic signature to which many humans are immunologically naïve. The human influenza virus genes contribute to the ability of the reassortant virus to replicate efficiently in human cells. It is thought that reassortment may take place in pigs that are dually infected with avian and human viruses. Currently, there is concern that a new pandemic strain of type A influenza could emerge as a derivative of highly virulent avian H5N1 influenza viruses now causing epidemics in domestic chickens in southeast Asia. As of January, 2006, there are over 100 documented human infections with the avian virus, mainly among poultry workers, with a mortality greater than 50%. However, few if any of these infections have spread from human to human, perhaps because the infecting avian virus has not undergone reassortment with a human influenza virus.

Virus strain	Origin of viral genes			Log 10 MLD 50	Log 10 virus titer in lung (day 3 after infection)
	HA	NA	Others		
M88	M88	M88	M88	>6.2	2.9
M88/H^{sp}	Sp (1918)	M88	M88	4.4	5.1
M88/H^{sp}/N^{sp}	Sp (1918)	Sp (1918)	M88	5.2	4.7

TABLE 15.4 Genetic determinants of the virulence of the 1918 type A influenza virus (Spanish strain) based on intranasal infection of mice with reassortant viruses. The M88 isolate is a human type A influenza virus which is relatively avirulent in mice, typical of human isolates. The hemagglutinin (HA) of the 1918 'Spanish' virus confers virulence upon the M88 isolate and the neuraminidase (NA) does not appear to enhance the effect of the hemagglutinin
MLD 50: mouse 50% lethal dose determined by intranasal infection with serial virus dilutions. After Kobasa D, Takada A, Shinya K *et al.* Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. *Nature*, 2004, 431: 703 707, with permission.

SIDEBAR 15.2

Speculative reconstruction of events following the transmission of SIVcpz to humans

This reconstruction is based on data in: Gao F, Bailes E, Robertson DL *et al.* Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. *Nature* 1999, 397: 436–441; Hahn BH, Shaw GM, De Cock KM, Sharp PM. AIDS as a zoonosis: scientific and public health implications. *Science* 2000, 287: 607–614; Nsilambi N, De Cock KM, Forthal DN *et al.* The prevalence of infection with human immunodeficiency virus over a 10-year period in rural Zaire. *New England Journal of Medicine* 1988, 318: 276–279.

Dates	Events
1915–1941	Transmission of SIVcpz to humans
~1930–1980	HIV-1 maintained in rural villages in Africa HIV and AIDS are not recognized
1980–1985	AIDS recognized and HIV-1 isolated
1980–present	HIV-1 spreads rapidly though some urban and rural populations in Africa Global spread of HIV-1 and AIDS

The 1918 pandemic of influenza is presumed to be an example of a zoonotic influenza virus that crossed the species barrier and became established in humans where it caused an excess mortality estimated at 20 to 40 million persons. Recent viral molecular archaeology has recovered the sequences of the 1918 H1N1 influenza virus from the tissues of patients who died during the epidemic. All of the genes of the reconstructed virus are avian in origin, but it is unknown whether the virus underwent mutations that enhanced its ability to be transmitted within the human population. The reconstructed hemagglutinin of the 1918 virus has been inserted into recombinant influenza viruses and, in mice, markedly increases the virulence of primary human isolates of influenza virus (Table 15.4), but the full virulence phenotype appears to require many of the avian influenza genes (see Table 9.14). Several physiological factors play a role in disease enhancement, including increased replication in pulmonary tissues and an enhanced ability to stimulate macrophages to secrete pro-inflammatory cytokines which, in turn, causes severe pneumonitis.

Human immunodeficiency virus (HIV)

HIV has emerged as the greatest pandemic in the history of medical science. A provisional reconstruction has been made of the sequence of events by which HIV appeared as a virus new to the human species (Sidebar 15.2). There were several transmissions of simian immunodeficiency virus (SIV cpz) of chimpanzees to humans, either by direct contact with captive chimpanzees or from the blood of slaughtered animals. Using a molecular clock, the original transmissions to humans are estimated to have occurred around 1930. The period from 1930 to 1980 is a mystery, but there are fragmentary data suggesting that the virus persisted as a rare and unrecognized infection in jungle villages in west Africa during this time. It has also been proposed that the re-use of unsterilized needles – a frequent practice during this period – could have inadvertently helped to spread the virus. Starting about 1980, the virus began to spread more rapidly, most frequently by the heterosexual route and transmission in urban settings was enhanced by urbanization and other social and economic displacements.

Did HIV undergo critical mutations in order to emerge as an established virus of humans? Available data are not sufficient to answer the question. However, there are many genetic differences between isolates of SIV cpz and ancestral clones of HIV. Also, HIV does not cause AIDS in most chimpanzees that have been experimentally infected and cultured chimpanzee T lymphocytes are not as permissive for HIV as are human cells. Thus, it is certainly possible that there were some critical mutations that were associated with the establishment of HIV-1 as a human virus.

Canine parvovirus (CPV)

Canine parvovirus is another example of a disease that emerged due to the appearance of a virus new to its host species. In the late 1970s, a highly lethal pandemic disease appeared in the dog populations of the world. The etiologic agent was a parvovirus previously unknown in canines. The sequence of canine parvovirus is almost identical to that of feline panleukopenia virus (FPV), an established parvovirus of cats, which causes acutely fatal disease in kittens. Canine parvovirus has a few point mutations that distinguish it from FPV and these mutations

permit CPV to bind to and infect canine cells, a property not possessed by FLV. It is thought that these mutations led to the emergence of a new virus disease of dogs.

The species barrier and host defenses

Viruses vary in their ability to cross the species barrier. Many mammalian viruses have evolved with their hosts so that, in nature, different members of a virus family are associated with each host species. Furthermore, under natural circumstances, each member of the virus family usually 'respects' the species barrier and does not cross into other species, although it spreads readily between individual animals within its host species. Undoubtedly, diverse mechanisms contribute to the species barrier. Recently, it has been recognized that specific host defenses and viral virulence genes play a role in species specificity. For instance, myxoma virus, a poxvirus whose natural hosts are various rabbit species, can replicate well in rabbit fibroblasts but not in mouse fibroblasts. The restriction in mouse cells is mediated by the intracellular cascade that activates type 1 IFN and genetic knockouts in the host that interrupt this signaling pathway render mouse cells fully permissive for myxoma virus. Conversely, poxviruses have a number of virulence genes that determine their host species range. Some of these viral genes act by interrupting host defenses, such as IFN induction pathway. Such viral genes may be host specific and engineered poxviruses that carry a virulence gene from a different poxvirus may exhibit an altered species range.

WHY VIRAL DISEASES ARE EMERGING AT AN INCREASING FREQUENCY

Although difficult to document in a rigorous manner, it does appear that new virus diseases of humans (and perhaps of other species) are emerging at an increased tempo. There are a number of reasons for this trend.

Population growth and aggregation

The human population is growing inexorably and is becoming urbanized even faster. As a result, there are an increasing number of large crowded urban populations, which provide an optimal setting for the rapid spread of any newly emergent infectious agent.

Transportation

In the 19th century, it was noteworthy that someone could circumnavigate the globe in 80 days, but now it can be done in less than 80 hours. However, the incubation period of viral infections (several days to several months) has stayed constant. Someone can be infected and, within a single incubation period, arrive at any other site on earth. This enhances the opportunity for a new human virus to spread as a global infection before it has even been recognized, markedly increasing the opportunity for the emergence of a new disease. The same dynamics also apply to viral diseases of animals, which may be overlooked but have important economic and social consequences.

The SARS pandemic of 2002–2003, described above, is an example of how rapidly and widely a new virus disease can emerge and spread globally. In this instance, it is extraordinary that the disease was brought under control – and eradicated from the human population – by the simple methods of isolation, quarantine and respiratory precautions. Although conceptually simple, a heroic effort was required for success.

Agribusiness

In the last 25 years, agriculture has undergone a dramatic evolution with the development of 'agribusiness'. Food and food animals are now raised on an unprecedented scale and under very artificial and crowded conditions, where the proximity of many members of a single plant or animal species permits an infection to spread like wildfire. Furthermore, increasing numbers of plants, animals and food products are rapidly transported over large distances.

Foot-and-mouth disease (FMD)

FMD is caused by a picornavirus that infects cattle, sheep and pigs and produces widespread vesicular infection of skin and mucous membranes. Although FMD is often not fatal, diseased animals do not recover and are lost as sources of milk and meat; convalescent animals can be persistently infected and shed virus for months or years. During the acute phase infection (first 2 weeks), high virus titers are shed in aerosols, excreta and secretions. FMD virus (FMDV) is one of the most infectious viruses known and it is transmitted mainly by the respiratory route. It is very hardy and can be spread by close contact, by fomites (such as contamined clothing and shoes) and as an aerosol, over many miles. Many countries control FMDV by exclusion of the virus rather than by immunization, so that there are large populations of agricultural animals that are susceptible to infection. In such countries, there is a constant danger of importation by infected animals, contaminated meat products, or aerosols and a single importation can trigger a rapid, catastrophic epidemic.

The FMD epidemic of 2001 in the UK is an example (Figure 15.7). Infection (source unknown) appeared in February and spread over much of England and Wales, peaking in April by which time about 2000 livestock farms had been involved. Due to a vigorous program of culling animals on infected and adjacent premises, the epidemic was brought under control by May and the virus had been eradicated by fall, 2001. Although the number of animals reported with FMD was less than 10 000, the control program required the culling or loss of about 10 million pigs, sheep and cattle and the cost of the epidemic was estimated at greater than $15 billion.

Monkeypox

The international shipment of plants and animals can import viruses into new settings where they may lead to the emergence of unforeseen diseases. One example is

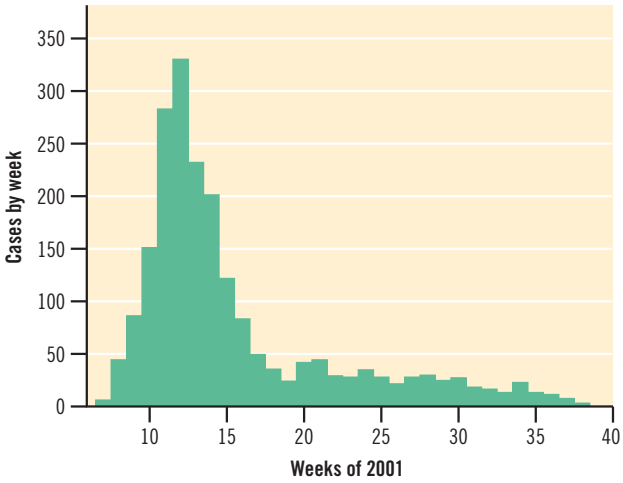

FIGURE 15.7 Reported cases of foot-and-mouth disease, UK, 2001. Cases are shown by day of report for infected premises. Once a farm was reported as an 'infected premise', all animals (cattle, sheep, pigs) were culled within 24–48 hours, so this is primarily a count of infected farms rather than infected animals. After graph on website of Department of Environment, Food and Rural Affairs, www.defra.gov.uk/footandmouth.cases.histogram, with permission.

the 2003 outbreak of monkeypox that caused about 80 human cases in the USA (Figure 15.8). This was traced to the importation from Africa of Gambian giant rats as exotic pets; several rodent species in west Africa appear to be reservoir hosts of this poxvirus. Monkeypox spread from these animals to pet prairie dogs and from prairie dogs to their owners.

Ecologic disruption by humans

Remote areas of the world are now being colonized at a high frequency, driven by economic motives, such as the reclamation of land for agriculture or other uses and the harvest of valuable trees and exotic animals. The construction of new dams, roads and other alterations of the natural environment create new ecological niches. It is possible that this is the origin of urban yellow fever, which is caused by an arthropod-borne flavivirus that was probably first transmitted to humans who entered jungle areas in South America and Africa where yellow fever virus is maintained in a monkey–mosquito cycle.

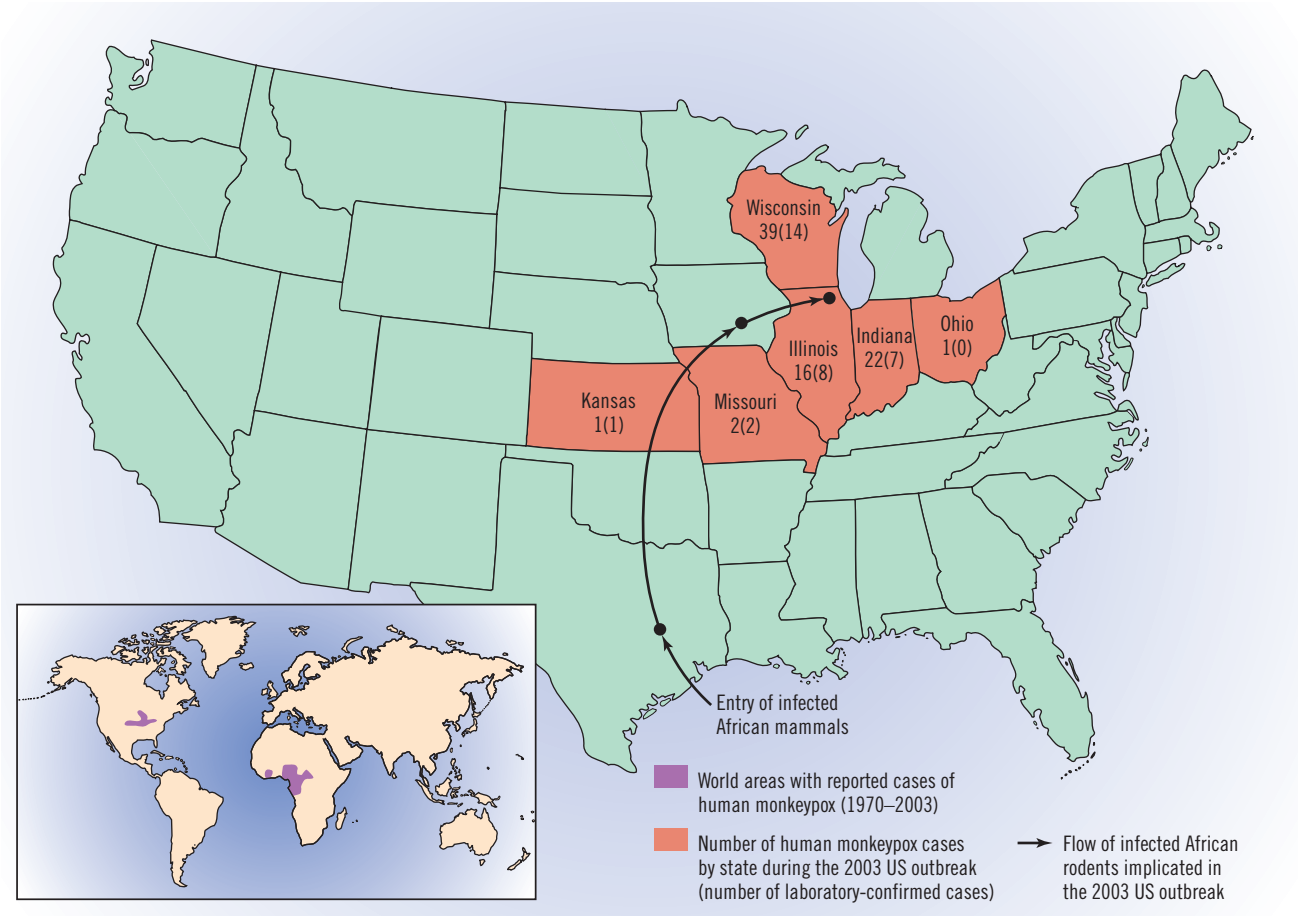

FIGURE 15.8 The distribution of cases of monkeypox, USA, 2003. Highlighted states show the numbers of cases of monkeypox and the arrows show the flow of infected African rodents implicated in the outbreak. After Di Giulio DB, Eckburg PB. Human monkeypox: an emerging zoonosis. *Lancet Infectious Diseases* 2004, 4: 15–25.

Deliberate introduction of a virus new to a specific population

Myxomatosis of rabbits

On occasion, a virus has been deliberately introduced into a susceptible population where it caused the emergence of a disease epidemic. For sport, rabbits were imported from Europe into Australia in the mid-19th century. Because of the absence of any natural predator, the rabbits multiplied to biblical numbers and threatened natural grasslands and agricultural crops over extensive areas of southern Australia. To control this problem, myxomatosis virus was deliberately introduced into southern Australia in 1950. This poxvirus is transmitted mechanically by the bite of insects and is indigenous to wild rabbits in South America, in which it causes non-lethal skin tumors. However, myxomatosis virus causes an acutely lethal infection in European rabbits and its introduction in Australia resulted in a pandemic in the rabbit population.

Following the introduction of myxomatosis virus in 1950, co-evolution of both virus and host were observed (Table 15.5). The introduced strain was highly virulent and caused epizootics with very high mortality. However, with the passage of time, field isolates exhibited reduced virulence and there was a selection for rabbits that were genetically somewhat resistant to the virus. Strains of moderate virulence probably became dominant because strains of lowest virulence were less transmissible and strains of maximum virulence killed rabbits very quickly.

Rabbit hemorrhagic disease

Rabbit hemorrhagic disease virus (RHDV), a calicivirus, can cause a fulminant form of liver failure and disseminated intravascular coagulation that kills in an extraordinarily short time (<48 hours) after infection. RHDV was first identified in China, in 1984, as the cause of an outbreak, originally thought to represent a newly emergent disease. However, it appears that RHDV has been enzootic in the UK and Europe for centuries, where it circulates widely and produces persistent infections with a low mortality. It has been suggested that the disease in Chinese rabbits was due to transmission following the importation of apparently normal but persistently infected rabbits from Germany. It is not clear why this virus can cause such acute illness in some circumstances and be relatively avirulent in other settings. Partial sequencing of genomes from both virulent and avirulent isolates has not yet revealed mutations associated with pathogenicity. Alternatively, it may be that the same virus can manifest as a relatively benign enzootic disease in populations where it has co-existed for a long time and as a virulent disease when it produces a pandemic in a naïve population. As described above, poliovirus exhibited two such different epidemiological patterns.

In 1997, RHDV was introduced into New Zealand by farmers who were desperate to control the proliferating rabbit population that was destroying pastures used to raise sheep. The virus strain was imported from Australia where it was highly lethal and it caused an extraordinary pandemic in the South Island of New Zealand. The rabbit population crashed but some rabbits survived, although the population did not return to its pre-epidemic density. There are no published data regarding the selection of genetic resistant rabbits or changes in the virulence of the introduced virus.

Concern has also been raised about disease re-emergence due to the deliberate introduction of viruses into either human or animal populations, as acts of bioterrorism.

Year	Virulence grade, fatality rate (%), mean survival time					No. of samples
	I >99 <13 days	II 95–99 14–16 days	III 70–95 17–28 days	IV 50–70 29–50 days	V <50 NA	
1950–51	100	0.0	0.0	0.0	0.0	1
1952–55	13	20	53	13	0.0	60
1955–58	0.7	5	55	24	15	432
1959–63	1.7	11	61	22	5	449
1964–66	0.7	0.3	64	34	1.3	306
1967–69	0.0	0.0	62	36	1.7	229
1970–74	0.6	5	74	21	0.0	174
1975–81	1.9	3	67	28	0.0	212

TABLE 15.5 The virulence of field isolates of myxoma virus between 1951 and 1981
Data taken from Fenner F. Biological control as exemplified by smallpox eradication and myxomatosis. *Proceedings of the Royal Society of London, part B Biological Sciences*, 1983, 218: 259–285, with permission.

Xenotransplantation

Because of the shortage of human organs for transplantation recipients, there is considerable research on the use of other species, particularly pigs, as organ donors. This has raised the question whether known or unknown latent or persistent viruses in donor organs might be transmitted to transplant recipients. Since transplant recipients are immunosuppressed to reduce graft rejection, they could be particularly susceptible to infection with viruses from the donor species. In the worst scenario, this could enable a foreign virus to cross the species barrier and become established as a new human virus that might spread from the graft recipient to other persons.

HOW ARE EMERGENT VIRUSES IDENTIFIED?

The impetus to identify a new pathogenic virus usually arises under one of two circumstances. First, a disease outbreak that cannot be attributed to a known pathogen may set off a race to identify a potentially new infectious agent. Identification of the causal agent will aid in the control of the disease and in prevention or preparedness for potential future epidemics. Classical methods may succeed in identifying the virus, or newer technology may be required. SARS coronavirus, West Nile virus in New York and Sin Nombre virus are examples of emergent viruses that were identified in the wake of outbreaks, using both classical and modern methods.

When a disease outbreak cannot be attributed to a known pathogen and where classical virus isolation and identification methods fail, the powerful and diverse methods of molecular virology may be called for. Researchers here too are not only driven by the importance of the disease, but also by the challenge of discovery. Each example of important non-cultivatable virus being discovered and characterized in recent years has involved different methods. Hepatitis C virus (HCV), Sin Nombre and other hantaviruses, certain rotaviruses and Kaposi's sarcoma herpesvirus (HHV8) are examples of emergent viruses that were first discovered as a result of elegant molecular technologies and carefully planned investigative strategies. Of course, an important new virus may be discovered as a serendipitous by-product of research directed to a different goal, as was the case with hepatitis B virus (HBV).

Methods of viral detection and identification are described briefly and more detailed information and technical specifics can be found in several current texts.

Classic methods of virus discovery

Is the disease infectious?

The first question that confronts the investigator faced with a disease of unknown etiology is whether or not it has an infectious etiology? Evidence that suggests an infectious etiology is an acute onset and short duration, clinical similarity to known infectious diseases, a grouping of similar illnesses in time and place and a history that suggests transmission between individuals presenting with the same clinical picture. For chronic illnesses, the infectious etiology may be much less apparent and a subject for debate.

Evidence of a viral etiology

Faced with a disease that appears to be infectious, the next question is whether it is caused by a virus. A classical example that predates modern virology is the etiology of yellow fever. In a set of experiments that would now be prohibited as unethical, the Yellow Fever Commission, working with US soldiers and other volunteers in Cuba in 1900, found that the blood of a patient with acute disease could transmit the infection to another person by intravenous injection. Furthermore, it was shown that the infectious agent could pass through a bacteria-retaining filter and therefore could be considered a 'filterable virus'.

Virus isolation in cell culture and animals

The first step in identification of a putative virus is to establish a system in which the agent can be propagated. Before the days of cell culture, experimental animals were used for this purpose. Many viruses could be isolated by intracerebral injection of suckling mice and some viruses that did not infect mice could be transmitted to other experimental animals. Human polioviruses, because of their cellular receptor requirements, were restricted to old world monkeys and great apes; the virus was first isolated in 1908 by intracerebral injection of monkeys and was maintained by monkey-to-monkey passage until 1949 when it was shown to replicate in primary cultures of human fibroblasts.

The modern era of virology (about 1950) can be dated to the introduction of cultured cells as the standard method for the isolation, propagation and quantification of viruses. There is now a vast range of cell culture lines that can be used for the isolation of viruses and, currently, this is the first recourse in attempting to isolate a suspected novel virus. Some viruses will replicate in a wide variety of cells but others are more fastidious and it can be hard to predict which cells will support their replication.

The introduction of cell culture for polioviruses opened the field to crucial experimental and epidemiological advances, which elucidated the pathogenesis of poliomyelitis and led to the development of inactivated poliovirus vaccine. In the course of these studies it was discovered that primary poliovirus isolates made in tissue culture were pantropic and could infect monkeys and chimpanzees by the oral route, replicating the natural route of human infection. In contrast, virus stocks from the pre-tissue culture era, which were propagated by monkey-to-monkey passage, were obligatory neurotropes and their use had misled researchers about the pathogenesis of the disease and the feasibility of a protective vaccine.

It is also important to recognize that some viruses will replicate in cell culture without exhibiting a cytopathic effect. An important example is the identification of simian virus 40 (SV40) as an exogenous agent in

harvests of poliovirus that were used for the production of inactivated poliovirus vaccine (IPV). Poliovirus was usually grown in primary cell cultures obtained from the kidneys of rhesus monkeys, but SV40 had escaped detection because it replicated without causing a cytopathic effect. When poliovirus harvests were tested in similar cultures prepared from African green monkeys, a cytopathic effect (vacuolation) was observed, leading to the discovery of SV40 virus in 1960. Because IPV produced from 1955 to 1960 had been prepared from virus grown in rhesus monkey cultures, many lots were contaminated with this previously unknown virus, which inadvertently had been administered to humans. Since that time, viral stocks and cell cultures have been screened to exclude SV40.

A number of methods are available to detect a noncytopathic virus that is growing in cell culture. These include visualization of the virus by electron microscopy, detection of viral antigens by immunological methods such as immunofluorescence or immunocytochemistry, the agglutination of erythrocytes of various animal species by virus bound on the cell surface (hemagglutination), the production of interferon or viral interference and the detection of viral nucleic acids, especially by PCR-based methods.

Electron microscopy

Electron microscopy is another established method for the detection of a virus. It can be used in conjunction with virus isolation or as the primary method to identify a virus that is not readily grown in cell culture or in experimental animals.

Example: rotaviruses In seeking the etiology of diarrheal diseases of infants, it was hypothesized that, in addition to bacteria, which accounted for less than half of the cases, one or more viruses might be responsible for some cases of infantile diarrhea. Numerous unsuccessful attempts were made to grow viruses from stools of patients with acute diarrhea. It was conjectured that it might be possible to visualize a putative fastidious virus by electron microscopy of concentrated fecal specimens. In 1973, Bishop and colleagues observed by electron microscopy, in the duodenal epithelium of children with diarrhea, a 70-nm virus, subsequently designated rotavirus. When patients' convalescent serum was added to filtered and concentrated stool specimens, aggregates of 70-nm virions were observed in stools from some infants with acute gastroenteritis. Individual virions were hard to observe, but addition of the antibody bound the virus particles into readily visualized aggregates. Also, the ability of convalescent, but not acute illness, serum to mediate virion aggregation provided a temporal association of the immune response with an acute diarrheal illness. In addition, stools from many infants with acute diarrheal disease yielded the same 70-nm virus particles while infants hospitalized at the same time for other illnesses were negative (Table 15.6).

These 70-nm particles soon were given the name rotaviruses. Immediate confirmatory reports from investigators in several different countries provided additional credibility to the provisional association of rotaviruses with infantile diarrhea, as did the antigenic relationship of the human viruses with animal rotaviruses that caused diarrhea in their respective host species. Within 5 years, rotavirus was recognized as the most common cause of diarrhea in infants and young children worldwide, accounting for approximately one third of cases of severe diarrhea requiring hospitalization.

The identification of the hemorrhagic encephalopathy (HER) strain of Kilham's rat virus provides another example where electron microscopy aided in the detection of a new viral disease. An investigator who was using chemical immunosuppression in an animal model of bone marrow transplantation observed that drug-treated control animals unexpectedly developed acute paralysis. Spinal cord tissues from paralysed animals yielded an agent that could be transmitted to suckling rats and electron microscopy of filtrates of the infectious material revealed a small virus, subsequently identified as a parvovirus of rats with an unusual ability to cause hemorrhagic encephalopathy.

Classification of a novel virus

Once an emergent virus has been identified, it is necessary to classify it, in order to determine whether it is a known virus, a new member of a recognized virus group, or represents a novel virus genus. This information provides clues relevant to diagnosis, prognosis, therapy and prevention. Prior to the era of genomics, classification was based on electron microscopy, identification of the viral nucleic acid (RNA or DNA, single or double stranded) and antigenic cross-reactivity with similar viruses.

Marburg virus In 1967, an outbreak of acute hemorrhagic fever occurred in laboratory workers who were harvesting kidneys from African green monkeys (*Cercopithecus aethiops*). In addition, the disease spread to hospital contacts of the index cases, with a total of over 30 cases and a 25% mortality. Clinical and epidemiological observations immediately suggested a transmissible agent, but attempts to culture bacteria were unsuccessful. However, the agent was readily passed to guinea pigs which died with an acute illness that resembled hemorrhagic fever. After considerable effort, the agent was adapted to tissue

Rotavirus particles in stool (70 nm by electron microscopy)	Complement fixing antibodies, median and (range)	
	Acute serum	Convalescent serum
Positive: 9 patients	<4(<4–8)	64 (32–>64)
Negative: 4 patients	<4(<4)	4 (<4–4)

TABLE 15.6 Rotavirus particles in stool and serological response in infants with acute gastroenteritis
After Kapikian AZ, Kim HW, Wyatt RG *et al.* Reovirus like agent in stools: association with infantile diarrhea and development of serologic tests. *Science* 1974, 185: 1049–1053, with permission.

SIDEBAR 15.3

The Henle-Koch postulates as updated by Evans

The Henle-Koch postulates were formulated in 1840 by Henle and revised by Koch in 1890, and have been modernized by many authors.

1. Prevalence of the disease should be significantly higher in those exposed to the putative cause than in control cases not so exposed.
2. Exposure to the putative cause should be present more commonly in those with the disease than in controls without the disease when all risk factors are held constant.
3. Incidence of the disease should be significantly higher in those exposed to the putative cause than in those not exposed, as shown in prospective studies.
4. Temporally, the disease should follow exposure to the putative agent with a distribution of incubation periods on a bell-shaped curve.
5. A spectrum of host responses should follow exposure to the putative agent along a biologic gradient from mild to severe.
6. A measurable host response following exposure to the putative cause should regularly appear in those lacking this before exposure or should increase in magnitude if present before exposure.
7. Experimental reproduction of the disease should occur in higher incidence in animals or humans appropriately exposed to the putative cause than in those not so exposed. This exposure may be deliberate in volunteers, experimentally induced in the laboratory, or demonstrated in a controlled regulation of natural exposure.
8. Elimination or modification of the putative cause or of the vector carrying it should decrease the incidence of the disease.
9. Prevention or modification of the host's response on exposure to the putative cause should decrease or eliminate the disease (e.g. immunization or drug).
10. The data should fit an internally consistent pattern that supports a causal association.

After Evans AS. Causation and disease: a chronological journey. *American Journal of Epidemiology* 1978, 108: 249–258. A further modification is presented in Fredricks DN, Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clinical Microbiology Reviews* 1996, 9:18–33.

culture and shown to be an RNA virus by virtue of its resistance to 5-bromodeoxyuridine (which inhibits DNA-dependent RNA synthesis and DNA viruses). When tissue concentrated culture harvests were examined by electron microscopy, it was immediately recognized that this agent differed from known families of RNA viruses, since the virions consisted of very long cylindrical filaments about 70 nm in diameter. This was the first recognized member of the filoviruses, which now include Marburg and Ebola viruses. These viruses have caused periodic outbreaks of severe hemorrhagic fever in central Africa. In 2005, one large outbreak in Angola was associated with a mortality rate over 80% and more than 300 deaths.

The Henle-Koch postulates

Isolation of a virus from patients suffering from an emergent disease provides an association, but not proof of a causal relationship. Formal demonstration that an isolated virus is the causal agent involves several criteria formulated over the past 100 years. These are often called the Henle-Koch postulates, after two 19th century scientists who first attempted to enunciate the rules of evidence. Sidebar 15.3 sets down these postulates, which have been modernized in view of current knowledge and experimental methods.

The classic version of the Henle-Koch postulates required that the causal agent be grown in culture. However, as discussed below, a number of viruses that cannot be grown in culture have been convincingly associated with a specific disease. Usually, this requires that many of the following criteria can be met:

1. Viral sequences can be found in the diseased tissue in many patients and are absent in appropriate control subjects.
2. Comparison of acute and convalescent sera document induction of an immune response specific for the proteins of the putative causal virus, evidence of infection preceding or concomitant with the occurrence of the disease.
3. The disease occurs in persons who lack a pre-existing immune response to the putative virus, but not in those who are immune.
4. The implicated virus or a homologous virus causes the disease in experimental animals.
5. Epidemiological patterns of disease and infection are consistent with a causal relationship.

Methods for detection of viruses that are difficult to grow in cell culture

It is now well established that some very important human diseases, such as hepatitis B and hepatitis C, are caused by viruses that cannot readily be grown in cell culture. These examples have had two important consequences:

1. they have given credibility to the view that an infectious etiology can be inferred by clinical and epidemiological observations in the absence of an identified causal agent.
2. they have stimulated researchers to devise novel techniques – for identification of a putative causal virus – that bypass the requirement for replication in cell culture.

Furthermore, the application of molecular biology, beginning about 1970, has led to an array of new methods, such as the polymerase chain reaction and genomic databases, that can be applied to the search for unknown viruses. Several case histories illustrate the inferences that lead to the hypothesis of a viral etiology, the strategy used to identify the putative causal agent and the methods exploited by ingenious and tenacious researchers.

Sin nombre virus (SNV)

Hantavirus pulmonary syndrome was described above as an example of an emerging virus disease. The disease was first reported in mid-May, 1993, and tissues and blood samples from these cases were tested extensively, but no virus was initially isolated in cell culture. However, when sera from recovered cases were tested, they were found to cross-react with a battery of antigens from known hantaviruses, providing the first lead (in June, 1993). DNA primers were then designed, based on conserved hantavirus sequences and these were used in a polymerase chain reaction (PCR) applied to DNA transcribed from RNA isolated from tissues of fatal cases. Sequence of the resulting amplicon suggested that it was a fragment of a putative new hantavirus (July, 1993), yielding a presumptive identification of the emerging virus within two months after the report of the outbreak. At this time the putative virus had not been propagated in cell culture, but an intense effort by three research teams led to the successful isolation of several strains of SNV by November, 1993. SNV is a fastidious virus that replicates in Vero E6 cells but not in a very large number of other cell lines that were tested.

Kaposi's sarcoma (KS) herpesvirus (HHV8)

KS was described over 100 years ago as a relatively uncommon sarcoma of the skin in older men in eastern Europe and the Mediterranean region. In the 1980s, KS emerged at much higher frequency as one of the diseases associated with AIDS. In the USA, its incidence in gay men is over 10 000-fold that in the general population and over 95% of cases occur in gay men with AIDS. Furthermore, KS exhibited an enigmatic epidemiological pattern, since its incidence in gay men was more than 10-fold greater than in other AIDS patients, such as injecting drug users and blood recipients. KS also occurs in women with AIDS and the prevalence among various HIV risk groups is highest in women who have bisexual male partners (Table 15.7). These observations led to a number of hypotheses regarding the etiology of KS, including the proposal that KS was caused by a previously undetected infectious agent that was more prevalent among gay men than among other HIV risk groups. However, researchers were unable to isolate a virus from Kaposi's sarcoma tissues.

Searching for footprints of such a putative agent, Chang and colleagues used the method of representational difference analysis (RDA) to identify DNA sequences specific for KS tumor tissue. Several DNA fragments were identified, shown to bear homology with sequences in known human and primate herpesviruses and used as probes to sequence the complete genome of a previously undescribed herpesvirus, since named HHV8, human herpesvirus 8 (Sidebar 15.4, Table 15.8). To this date, HHV8 defies cultivation in tissue culture.

The identification of HHV8 made it possible to develop tests for HHV8 antibodies and use these to re-examine the epidemiology of KS in light of HHV8 prevalence (Table 15.9). Seroepidemiological studies confirmed that HHV8 infection was much more frequent in gay men than in other HIV risk groups in the USA, explaining the differential incidence in different groups of AIDS patients and providing indirect support for the causal association of HHV8 and KS. Based on detection of viral DNA, HHV8 is shed in saliva but is infrequently found in

HIV risk group	Male partner	Proportion with KS	
		Number	Percent
Heterosexual contact	Bisexual	9/303	3.0
	IV drug user	9/1238	0.7
	Transfusion recipient or hemophiliac	0/71	0
IV drug user	Bisexual	3/74	4.0
	IV drug user	6/531	1.1

TABLE 15.7 The prevalence of Kaposi's sarcoma (KS) in women with AIDS, according to their sexual contacts
After Beral V, Peterman TA, Berkelman RL, Jaffe HW. Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? *Lancet* 1990, 335: 123–128, with permission.

SIDEBAR 15.4

Detection of DNA fragments of a novel herpesvirus in Kaposi's sarcoma tissue

'To search for foreign DNA sequences belonging to an infectious agent in AIDS-KS, we used representational difference analysis (RDA) to identify and characterize unique DNA sequences in KS tissue. . . The initial round of amplification-hybridization from KS and excess normal-tissue DNA. . .resulted in. . .four bands at approximately 380, 450, 540, and 680 base pairs. . .[which]. . . .became discrete after a third round of amplification-hybridization. The four KS-associated bands (designated KS330Bam, KS390Bam, KS480Bam, and KS631Bam). . .were gel purified. KS390Bam and KS480Bam Southern (DNA) hybridized nonspecifically to both KS and non-KS human tissues and were not further characterized. The remaining two RDA bands, KS330Bam and KS631Bam, were cloned and sequenced. K330Bam is 51% identical by amino acid homology to a portion of the ORF26 open reading frame encoding the capsid protein VP23 of herpesvirus saimiri. . .[and is]. . .also 39% identical to the amino acid sequence encoded by the corresponding BDLF1 ORF of Epstein-Barr virus. KS631Bam has homology to the tegument protein (ORF75) of herpesvirus saimiri and to the tegument protein of EBV (ORF BNRF1). To determine the specificity of KS330Bam. . .for AIDS-KS, these sequences were hybridized to Southern blots of DNA extracted from cryopreserved tissue obtained from patients. . .Although these sequences suggest the presence of a new human herpesvirus in KS lesions, a causal link between these sequences and AIDS-KS cannot be established by our retrospective case control study.'
From Chang Y, Cesarman E, Pessin Ms *et al.* Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 1994, 266: 1865–1869, with permission.

Patients	Tissue type	Positive by KS300 Bam DNA hybridization
AIDS	KS lesions	20/27 (74%)
AIDS	Lymphomas and lymph nodes	6/39 (15%)
Non-AIDS	Lymphomas and lymph nodes	0/36 (0%)

TABLE 15.8 Relative frequency of DNA sequences of a putative new herpesvirus in tissues from AIDS patients with Kaposi's sarcoma and non-AIDS patients
After Chang Y, Cesarman E, Pessin MS *et al.* Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 1994, 266: 1865–1869, with permission.

HIV status	HIV risk group	Kaposi's sarcoma	Antibodies to HHV8 (% positive)
HIV positive	Gay men	KS	84/104 (82)
	Gay men	No KS	10/33 (30)
	Hemophiliac	No KS	0/26 (0)
	IV drug users	No KS	0/38 (0)
HIV negative	Gay men	No KS	8/65 (12)
	IV drug users	No KS	0/25 (0)

TABLE 15.9 Frequency of antibodies to HHV8 in diverse populations. (The immunofluorescent antibody test used was less than 100% sensitive)
After Simpson GR, Schulz TF, Whitby D *et al.* Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibody to recombinant capsid protein and latent immunofluorescence antigen. *Lancet* 1996, 349: 1133–1138, with permission.

semen, suggesting that it is probably transmitted mainly by oral-oral and oral-genital routes. Consistent with this view, antibodies to HHV8 are often acquired during childhood in HHV8-high-prevalence regions such as sub-Saharan Africa.

Kawasaki disease

Kawasaki disease is an acute vasculitis of children which can cause aneurysms of the coronary arteries and other vascular pathologies and is usually preceded by fever, rash and lymphadenopathy. Kawasaki disease can occur in geographic clusters that are most frequent in winter and spring. These clinical and epidemiological descriptors have suggested that Kawasaki disease may be caused by a virus. A number of putative viral etiologies have been proposed but none of these associations has been consistently confirmed.

Recently, a group of investigators decided to search for human coronaviruses as a possible cause of respiratory illness in young children. They were motivated by the observation that many acute respiratory illnesses cannot be associated with a specific infectious agent and coronaviruses are an important cause of respiratory illness in several animal species. To search for a putative virus, the researchers compared known avian and mammalian coronaviruses and identified a highly conserved sequence in the viral replicase (an RNA-dependent RNA polymerase). For PCR tests, primers were synthesized that would detect this conserved sequence and were applied to respiratory specimens obtained from young children with acute respiratory infections. From a few specimens an amplicon was obtained that had a sequence that was partly homologous with several known coronaviruses and which was interpreted to be part of the genome of a novel human coronavirus, designated as HCoV-NH (for New Haven). Based on this novel sequence, a second set of primers was used to screen respiratory samples from 895 young children with acute respiratory illnesses and almost 10% tested positive for HCoV-NH. This 'new' virus was similar if not identical to another recently identified human coronavirus, that was also associated with respiratory illness.

One child who was positive for HCoV-NH developed Kawasaki disease and led the investigators to consider the hypothesis that HCoV-NH might be one cause of Kawasaki disease. To test this hypothesis, they conducted a small case control study: eight of 11 cases of Kawasaki disease and only one of 22 controls were positive for HCoV-NH. At this writing (January, 2006), it is premature to conclude that one cause of Kawasaki disease has been found and several recent published notes indicate that other investigators have failed to confirm these findings. However, the story provides an instructive example of the problems in identifying novel pathogenic viruses of humans that are not readily isolated in cell culture.

REPRISE

New virus diseases of humans and animals continue to emerge, even though the science of virology has been well established for more than 50 years. There are several explanations for emergence:

1. discovery of the cause of a recognized disease
2. increase in disease caused by an existing virus due to changes in host susceptibility or in virus virulence
3. re-introduction of a virus that has disappeared from a specific population
4. a virus that crosses the species barrier into a new species previously uninfected. Many zoonotic viruses that are maintained in a non-human species can infect humans, but most cause deadend infections that are not transmitted between humans. A few zoonotic viruses can be transmitted between humans but most fade out after a few person-to-person transmissions. Rarely, as in the case of HIV or SARS coronavirus, a zoonotic virus becomes established in humans, causing a disease that is truly new to the human species.

There are many reasons for the apparent increase in the frequency of emergence of new virus diseases, most of which can be traced to human intervention in global ecosystems. Emergent viruses are identified using both classical methods of virology and newer genome-based technologies. Once a candidate virus has been identified, a causal relationship to a disease requires several lines of

evidence that have been encoded in the Henle-Koch postulates, guidelines that are periodically updated as the science of virology evolves.

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Chapter 16

Antiviral Therapy

Neal Nathanson and Douglas D. Richman

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REPRISE

FURTHER READING

Although the last 25 years has witnessed an explosion of knowledge in the field of virology, the development of effective antiviral therapeutics, with the exception of HIV, has not kept pace. However, the credibility of antiviral therapy was established many years ago by a limited number of approved treatments for specific viral infections and has been reinforced, since 1990, by the large and constantly growing number of effective anti-HIV drugs. In this chapter we will focus on the mechanisms by which antiviral agents act, illustrated by selected examples. In addition, we attempt to highlight the importance of viral pathogenesis for designing different therapeutic strategies for individual viral diseases. The chapter concludes with a brief overview of those virus infections for which the most effective therapy is available. More comprehensive information on antiviral therapy is presented in several recent books and reviews.

PRINCIPLES OF ANTIVIRAL THERAPY

Antiviral therapeutics can be divided into several categories: drugs that are directed against the virus itself (either its genome or its proteins);

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Variable	Enfuvirtide (N = 335)	Control (N = 169)	Difference between the two groups
Mean change from pre-treatment level (log 10 HIV RNA copies per ml)	−1.429	−0.648	0.781
Percent of patients with <50 HIV RNA copies per ml	12.2	5.3	6.9
Percent of patients with <400 HIV RNA copies per ml	28.4	13.6	14.8
Percent of patients with >10-fold decrease in HIV RNA copies per ml	42.7	20.7	22.0

TABLE 16.1 Efficacy of an HIV-1 entry inhibitor for treatment of HIV-infected patients. In this study, patients who had 'failed' treatment with other anti-HIV drugs were put on optimal treatment regimens and divided into two groups, controls and those who were treated with enfuvirtide (T20, an entry inhibitor) in addition. Both groups were compared after 24 weeks to determine the level of HIV RNA in their plasma. The differences shown were statistically significant ($p < 0.01$)
After Lazzarin A, Clotet B, Cooper D *et al.* Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. *New England Journal of Medicine* 2003, 348: 2186–2185, with permission.

drugs that are directed against the proteins of the host cell that are critical for the replication of individual viruses; and therapeutics that mimic or enhance host defense mechanisms.

Virus as target

Viral proteins

Current understanding of the molecular replication of individual viruses has, in a few instances, provided a detailed elucidation of the role of individual viral proteins. It is possible to map functional domains within viral proteins and to image their structures. These data can be used for 'rational' drug design, either to synthesize small molecules that will bind to active sites on viral proteins, or to develop high throughput screening procedures to test a very large battery of small molecules for those that block a specific activity.

The gp41 protein of HIV-1 is an example. The sequential steps in entry of HIV-1 are described in Chapter 14 (see Figure 14.2). After binding of HIV gp120 to the cellular coreceptor (CCR5 or CXCR4), HIV gp41 undergoes a conformational change that exposes its N terminus fusion domain, which inserts into the plasma membrane of the host cell. Close to the N terminus of gp41 is a heptad repeat (HR1) that forms a three-helix bundle. In the next step in entry, this bundle associates with another three-helix bundle composed of heptad repeats (HR2) at the C terminus of gp41; this association forces the molecule into a hairpin configuration. A synthetic oligopeptide analog of HR2, enfuvirtide (originally called T20), can bind to HR1 and prevent hairpin formation, thereby blocking HIV-1 cellular entry. Enfuvirtide has been shown to be active in HIV-1 infected patients who have 'failed' other anti-HIV drug therapy (Table 16.1).

Viral mutagens

The survival of viruses as life forms depends in part on their ability to evolve in response to antiviral pressures, such as host immune responses. From this perspective, the rapid mutational rate of RNA viruses, in particular, facilitates the selection of fitness mutants among an ever-present swarm of genetic variants. In theory, the polymerases of RNA viruses may have evolved to an optimal balance of processivity and mutational rate, which permits the generation of large numbers of progeny with many genetic variants. These variants facilitate rapid adaptation to selective pressures such as immune responses and drug treatment. One novel approach to antiviral drugs is the use of mutagens that can increase viral mutational rate, so that fit variants are overwhelmed by less fit or nonsense mutants.

Although this strategy for development of antiviral agents may be counterintuitive, it appears that at least one antiviral drug may act through this mechanism. Ribavirin, a nucleoside analogue, has activity against a number of RNA viruses, but its mechanism of activity has been debated. The drug acts as an RNA mutagen and there are experimental data suggesting that it can push an RNA virus into 'error catastrophe' (Figure 16.1).

RNA interference (RNAi)

In the mid-1990s, RNA interference was discovered serendipitously when an attempt to over-express specific plant genes, using viral vectors, instead resulted in the knockout or silencing of those genes. The mechanism has now been elucidated to some extent (Figure 16.2). Double-stranded RNA is synthesized as an essential intermediate in the replication of most viruses. Eukaryotic organisms have a ribonuclease III (class of ribonucleases specific for double-stranded (ds) RNA) that cleaves

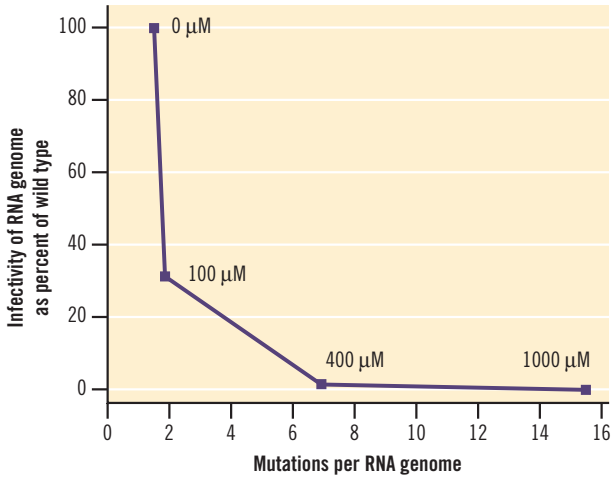

FIGURE 16.1 Ribavirin can initiate RNA 'error catastrophe' in a model virus. Poliovirus was grown in the presence of increasing doses of ribavirin and the infectivity per genome was plotted against the number of mutation per genome. The concentration of ribavirin is shown for each point. After Crotty S, Cameron CE, Andino R. RNA virus error catastrophe: direct molecular test by using ribavirin. *Proceedings of the National Academy of Sciences* 2001, 98: 6895–6900.

dsRNA into small ds oligonucleotides of 21–25 base pairs (the first member of this enzyme family was described in Drosophila and named 'DICER'). DICER and its homologs act to protect host cells against foreign RNAs, including those generated by invading viruses. The oligonucleotides produced by DICER homologs act as small interfering RNAs (siRNAs) by forming RNA-induced silencing complexes (RISCs) which, with the participation of helicase, unwind the ds oligonucleotides. The resultant single-strand (ss) oligonucleotides guide the RISC to homologous RNAs, which are then degraded.

RNAi has been adapted for the experimental silencing of specific genes and is being developed as a potential antiviral therapeutic drug. Theoretically, siRNAs could be used to silence viral genes or host genes essential for viral replication. Using cell cultures, siRNA has been introduced by a variety of methods (such as expression by a lentivirus vector) and has shown to have the ability to markedly reduce replication of HIV-1 and other viruses. Of note, long-term RNAi treatment of cell cultures selected for HIV escape mutants, which both validates the effect of siRNA and underlines its therapeutic limitations for persistent infections.

The delivery of siRNAs in vivo would be a major challenge. Somewhat surprisingly, siRNA itself can induce protection in mice, albeit under somewhat artificial conditions (Table 16.2). However, if RNAi is to be developed for human therapy it would have to be delivered by viral vectors or other strategies that would have minimal toxicity and would enable continual synthesis or release of therapeutic oligonucleotides.

Antiviral peptides

Plants and animals produce 'natural' antimicrobial peptides that provide a defense against bacterial and fungal

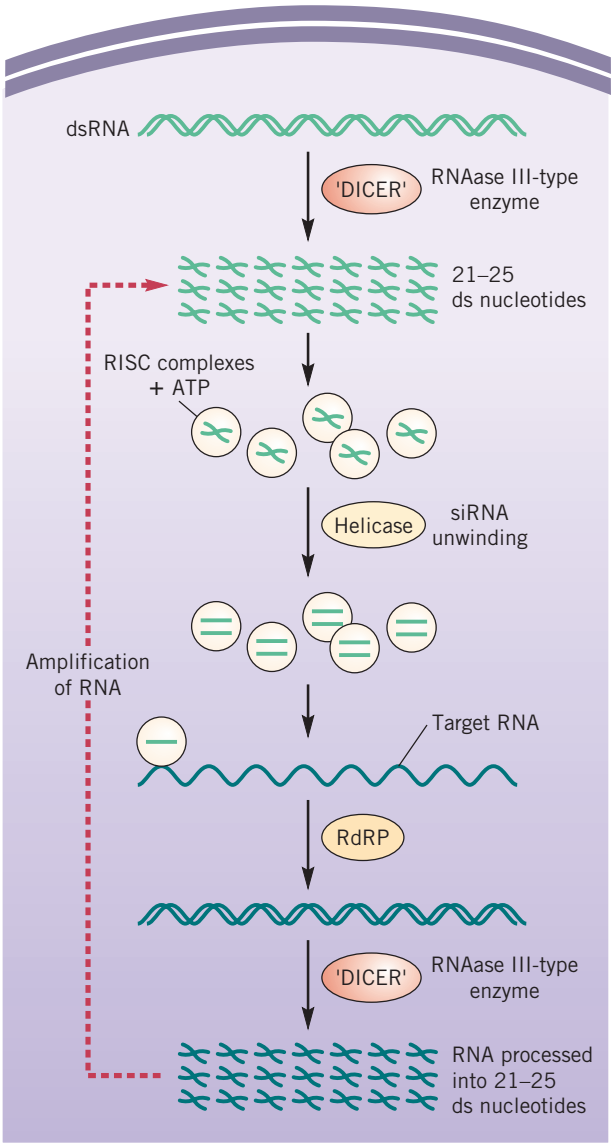

FIGURE 16.2 Diagram of RNAi (RNA interference). Synthesis of double-stranded RNA (dsRNA) is an essential step in the replication of most viruses. dsRNA triggers the action of an RNAse III-type enzyme (homolog of the Drosophila enzyme 'DICER') that cleaves the dsRNA into ds 21–25 nucleotides. These oligonucleotides act as small interfering RNAs (siRNAs) by forming RNA-induced silencing complexes (RISCs) which unwind the siRNAs into single stranded molecules. These ssRNAs guide the complex to bind to homologous ssRNAs which are then converted into dsRNAs by RNA-dependent RNA polymerase (RdRP); the nascent dsRNA is then degraded by DICER homologs, effectively silencing the corresponding gene. After Dave RS, Pomerantz RJ. RNA interference: on the road to an alternate therapeutic strategy! *Reviews in Medical Virology* 2003, 13: 373–385.

infections. Most of these molecules are amphipathic, composed of discrete hydrophobic and cationic (positively charged) domains. Some of these natural peptides, such as magainin and some defensins, have activity against selected viruses, such as herpesviruses (HSV) 1 and 2. The primary receptors for HSV-1 and HSV-2 are heparin sulfate and other negatively-charged glycosoaminoglycan chains on the surface of susceptible cells. The antimicrobial peptides apparently protect by binding to these negatively charged receptors, thereby

Treatment	Log_{10} virus titer in lungs	Fold reduction in titer	Percent mortality at 3 weeks
siGFP (control)	5.6	0	60
siNP	3.8	63	20
siPA	4.6	10	10
siNP + siPA	3.8	63	0

TABLE 16.2 RNAi protects against influenza virus in a mouse model. Mice were treated with an siRNA that targeted a specific gene of influenza virus (or an irrelevant gene) and then infected intranasally with a mouse-virulent type A H1N1 strain of influenza virus. Virus titers in the lung were measured 2 days after infection and cumulative mortality was recorded 3 weeks after infection
NP: nucleoprotein; PA: acid polymerase; GFP: green fluorescent protein (control). After Tompkins SM, Lo C-Y, Tumpey TM, Epstein SL. Protection against lethal influenza virus challenge by RNA interference in vivo. *Proceedings of the National Academy of Sciences* 2004, 101: 8682–8686, with permission.

SIDEBAR 16.1

Determinants of antiviral drug resistance

- Variation in viral mutation rate, which is about 10 000-fold greater for RNA than DNA viruses.
- Variation in in vivo viral replication, which determines the rate at which mutants are generated.
- Variation in the structural mechanism of drug-mediated viral inactivation, which determines the frequency and fitness of resistant mutants.
- Variation in drug-mediated selective pressure in vivo, which determines the relative replication rates of wildtype and mutant viruses.
- Concurrent use of several drugs that act upon different viral functions will markedly reduce the frequency of resistant virions, since these must possess multiple mutations.

blocking viral attachment. Also, it appears that, upon stimulation, human CD8+ T lymphocytes secrete α-defensins that account in part for their long-known anti-HIV-1 activity. In contrast to herpesviruses, the anti-HIV activity of α-defensin is due to a post-entry block in viral replication, yet to be defined. It is not clear whether the naturally occurring antimicrobial peptides have activity against a wider group of virus families, or whether synthesized congeners of these antimicrobial peptides might be developed as antiviral therapeutic drugs.

Drug-resistance mutations

Drug-resistant viral mutants constitute a major problem in antiviral therapy. The frequency of resistant mutants varies widely and is determined by a number of factors (Sidebar 16.1).

1. RNA viruses have a mutation rate estimated at 10^{-4} (1 mutation in 10 000 base-replications) that is much higher than the rate for DNA viruses (10^{-8}); the difference reflects the absence of cellular proof-reading mechanisms for RNA.
2. The replication rate of the virus during a specific infection will vary widely and influence the rate at which mutant virions are produced. For instance, it has been estimated that during an HIV-1 infection, 10^8 to 10^{11} virions are produced daily; this would yield 10^4 to 10^7 virions with single point mutations (or an average 1 to 1000 mutants for each of the 10 000 bases) each day. At the other end of the scale, human papilloma virus (HPV, a DNA virus) replicates very slowly in vivo, so that very few mutant virions would be synthesized daily. These differences are reflected in the observation that individual primary HIV isolates consist of a 'swarm' of viruses the sequences of which, after several years of infection, vary from 5 to 10%, for different genes. Primary isolates of DNA viruses show much less variation.
3. Different classes of drugs target diverse viral functions that vary in their importance for viral replication and individual drugs vary in the degree to which they can block their targeted function. Furthermore, resistant mutants vary in their ability to replicate in the presence of the drug and also, absent drug, in their replicative capacity or fitness, due to the structural differences that are required to escape binding by the antiviral compound. These nuances are reflected in the observation that different HIV-1 nucleoside reverse transcriptase inhibitors (NRTIs), which are directed against the same viral function, select for different escape mutations.
4. The in vivo selective pressure of a specific drug will depend upon both its intrinsic ability to block an essential virus function and its pharmacodynamics, which will determine its actual concentration at sites of viral replication. As the selective pressure increases, the relative advantages of mutants increase, but the rate of replication of wild type virus decreases thereby reducing the frequency of mutants. These two opposing effects create an 'optimum' drug concentration at which the selection of escape mutants is maximized.

One important implication of the foregoing considerations is the potential advantages of multidrug therapy. If a virus has to replicate in the presence of three diverse drugs, each of which select resistant mutants at a frequency of 10^{-5}, then triple mutants (assuming no interaction

Number of drugs (Number of patients)	Relative blood HIV RNA concentration according to days after beginning antiretroviral therapy		
	0 days	7 days	28 days (~plateau level)
1 (N = 16)	100%	30%	18%*
2 (N = 15)	100%	6%	1.4%*
4 (N = 16)	100%	4%	1.0%
5 (N = 9)	100%	2%	0.3%*

TABLE 16.3 Effect of multiple drug therapy on decrease in plasma levels of HIV RNA. HIV-infected treatment-naïve patients were started in regimens of 1, 3, 4 or 5 antiretroviral drugs and the decrease in the level of blood HIV RNA was determined for the first 7–28 days of therapy
* Extrapolations, not actual measurements. After data in Grossman Z, Polis M, Feinberg MB *et al.* Ongoing HIV dissemination during HAART. *Nature Medicine* 1999, 5: 1099–1103, with permission.

between various mutations) would occur at 10^{-15}, which might be a very rare phenomenon. In the case of HIV-1, there has been a comparison of multiple drug therapy as new compounds have been introduced. There is a dramatic stepwise increase in efficacy with each additional drug (Table 16.3). The optimal treatment strategy is to completely suppress viral replication which could prevent the emergence of resistant mutants.

Host cell as target

Cellular proteins

All viruses depend on the participation of many proteins provided by the host cells in which they replicate. The enumeration of this large array of cellular proteins has not yet been completed for any single virus. Nevertheless, a partial inventory does provide an additional set of potential targets for antiviral drugs. For long-term therapy, it would be necessary to avoid interfering with vital cellular functions, but for acute infections this might not be a critical impediment.

One example is CCR5 (chemokine receptor 5), the coreceptor for HIV (see Chapter 3). It happens that some apparently normal humans are homozygous for a mutation in CCR5 (the 'Δ32' mutation) that prevents the expression of this host gene; furthermore, such individuals are very resistant to HIV infection (see Chapters 13, 14). These observations suggest that a small molecule that blocked the receptor domain on CCR5 might be used to treat HIV infection without interfering with any essential cellular functions. Figure 16.3 demonstrates the potential ability of chemokine mutants that bind CCR5 to down modulate HIV-1 infection.

An experimental model, in which vaccinia virus causes lethal pneumonitis in mice, illustrates the potential of therapies directed against cellular proteins. ErbB-1, a cellular growth factor receptor, is known to play a role in the release of poxviruses from infected cells. An inhibitor of ErbB-1 had a dramatic ameliorating effect that was enhanced by simultaneous treatment with a neutralizing antibody against vaccinia virus (Table 16.4). Likewise, it has been shown that Gleevec, a drug approved

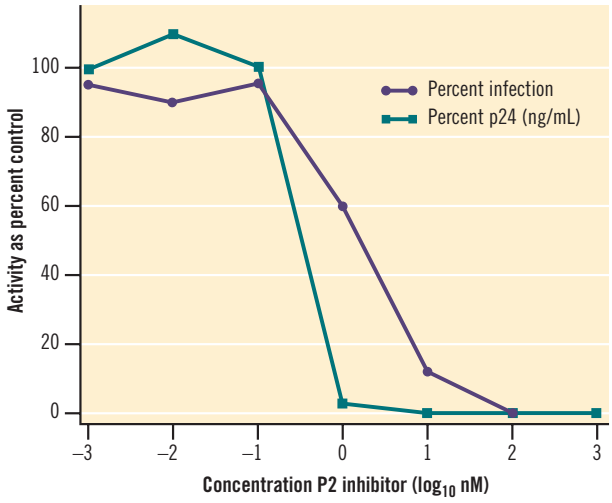

FIGURE 16.3 A chemokine-like molecule that binds to the CCR5 coreceptor can inhibit HIV-1 entry and infection of human cells. P2, a mutant of RANTES (a chemokine that binds to CCR5, chemokine receptor 5) was used to treat cells during infection. Two different experiments are shown. Per cent infection: macrophage-derived monocytes were subjected to a single round of infection with non-replicating HIV-1 particles that expressed a luciferase protein and the per cent luciferase positive cells was determined at 48 hours. Per cent p24: activated PBMCs (peripheral blood mononuclear cells) were treated with various concentrations of P2 inhibitor and were infected with a wildtype HIV-1 isolate and p24 levels in the supernate were measured. For both experiments data are plotted as per cent of control infections in the absence of P2 inhibitor. After Hartley O, Dorgham K, Perez-Bercoff D *et al.* Human immunodeficiency virus type 1 entry inhibitors selected on living cells from a library of phage chemokines. *Journal of Virology* 2003, 77: 6637–6644.

for the treatment of chronic myeloid leukemia, can reduce vaccinia mortality in mice. Gleevec is an inhibitor of cellular tyrosine kinases, which are required for the release of poxviruses (cell-associated enveloped virions) from the infected cell, explaining its antiviral activity.

Viral pathogenesis and antiviral strategy

The pathogenesis, transmission and epidemiological characteristics of individual viruses are important determinants of the potential efficacy of antiviral drugs.

Treatment group	Survival (%)	Lung weight (g) 6 days after infection (normal = 0.22)	Virus titers (log_{10} PFU per lung) 8 days after infection
None	0	0.47	7.5
CI-1033	100	0.40	5.9
Anti-L1R	80	0.38	4.3
CI-1033 + anti-L1R	100	0.26	<1.0

TABLE 16.4 Drug inhibition of a cellular protein ameliorates lethal vaccinia virus pneumonia in mice. All mice were infected with a lethal intranasal dose of vaccinia virus and subjected to various treatments. CI-1033 is an Erb B inhibitor and anti-L1R is a vaccinia virus neutralizing monoclonal antibody
After Yang H, Kim S-K, Kim M *et al.* Antiviral chemotherapy facilitates control of poxvirus infections through inhibition of cellular signal transduction. *Journal of Clinical Investigation* 2005, 115: 379–387, with permission.

Age at initial infection	Outcome	Relative frequency (%)
Normal adults	Inapparent or anicteric disease	65–80
	Icteric disease	20–35
	Mortality	0.2–0.5
	Complete spontaneous recovery and viral clearance	90–98
	Viral persistence and chronic hepatitis	2–10
Newborn infants	Inapparent, with icteric or anicteric disease	10–30
	Icteric disease	<5
	Complete spontaneous recovery and viral clearance	10–30
	Viral persistence and high risk of hepatocellular carcinoma	70–90

TABLE 16.5 Course of infection with hepatitis B virus (HBV). The substantial frequency of spontaneous recovery and viral clearance suggests that persistent HBV infection might be a good candidate for therapy
After Hollinger B, Liang TJ. Hepatitis B virus, chapter 87 in Knipe DM, Howley PM (eds), *Fields Virology*, 4th edn, 2001, with permission.

Viruses that have a very short incubation period and generation time and spread very rapidly tend to be poor candidates for antiviral treatment because it is difficult to complete diagnosis and initiate therapy in a timely fashion. Influenza is a good example of a serious illness with a short incubation period (18–72 hours); neuraminidase inhibitors are quite effective anti-influenza drugs but need to be given prior to infection or very soon after symptoms appear. This drawback may be overcome under certain circumstances; in the presence of a pandemic wave of influenza that is spreading across a community, antiviral drugs could be widely administered as a short-term prophylactic, thereby anticipating potential infection.

Persistent viral infections are often appropriate targets for antiviral treatment because they may be associated with significant chronic illness. Also, an accurate diagnosis and evaluation can be made prior to initiating therapy. Furthermore, there are a few persistent infections, such as hepatitis B and hepatitis C, which have infected a large number of humans (>500 million globally) who are at long-term risk of serious or fatal illness, such as liver failure or hepatocellular cancer. Effective therapeutic intervention, particularly if it resulted in long-lasting viral suppression or viral clearance (a 'cure'), could significantly reduce morbidity and mortality.

In many persistent infections, there is a dynamic balance between the persistent virus and host defenses. Thus, certain hosts are able to clear the infection even after years of persistence (Table 16.5). A pathogenesis pattern of this type has two implications for antiviral therapy. First, it suggests that antiviral therapy might tip the balance in favor of the host and lead to complete

Days after infection	Parameter	Control animals	Antibody treatment
Day 5 prior to treatment	Virus titer in brain (median $\log_{10}$ PFU per g)	4.0	4.0
Day 9	Virus titer in brain (median $\log_{10}$ PFU per g) (range)	5.0 (3.0–8.0)	2.0 (neg–6.0)
Day 30	Survival	20%	80%

TABLE 16.6 Therapeutic effect of neutralizing monoclonal antibody against West Nile virus infection of the central nervous system. Mice were infected subcutaneously with 100 PFU and were treated intraperitoneally with 2 mg of antibody on day 5 of infection, when the virus was already established in the brain. The antibody treatment markedly improved survival and also accelerated clearance of virus from the brain
After Oliphant T, Engle M, Nybakken GE *et al.* Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. *Nature Medicine* 2005, 11: 522–530, with permission.

viral elimination and second, it suggests that antiviral antibodies could be used in synergy with antiviral drugs to improve the therapeutic outcome.

Therapeutic antibody

Neutralizing antibodies are a major mediator of the pre-exposure protection conferred by many established viral vaccines (see Chapter 17). In addition, antibodies induced during primary infection play a role in clearance and recovery from certain acute viral diseases (see Chapter 6). Therefore, it is plausible that passive antibody administered during acute viral infection might be therapeutic.

Primary infection with West Nile virus (WNV), a flavivirus, is one example. Following transmission by mosquito bite, WNV initiates a plasma viremia followed by invasion of the central nervous system, resulting in potentially fatal encephalitis. Experiments in immunologically deficient mice have shown that both antibody and cellular immunity play a role in the outcome of infection. Neutralizing antibody, particularly IgM, can abort viremia and prevent WNV invasion of the CNS, while CD8+ T cells are required for effective clearance of virus from infected tissues. Surprisingly, antibody also plays a role in the clearance of virus from the central nervous system, even when infection has been well established in neurons. When administered to mice undergoing acute WNV encephalitis, passive antibody can markedly improve survival, at least under experimental conditions (Table 16.6).

Interferons (IFNs) and interferon inducers

Type 1 interferons (IFN-$\alpha$and β) are an important component of the innate immune response (discussed in Chapter 5). IFNs induce a complex pleiotropic response that inhibits viral replication in several different ways, in addition to synergizing antigen-specific acquired immune responses. Use of exogenous IFN as an antiviral therapy has been tried for many viral infections but, to date, has been adopted mainly for the treatment of two persistent infections, HCV and HBV (discussed below). In part, this is due to the expense of IFN therapy and to its substantial side effects in humans.

Pharmacodynamics, bioavailability and toxicity

Although beyond the scope of this text, it should be mentioned that, in addition to antiviral activity, potential drugs must meet a daunting set of criteria regarding their pharmacological properties. These include: route of administration to patients, distribution in bodily tissues and fluids, ability to reach virus-infected target tissues, activation or degradation in the liver and other tissues and dynamics of degradation or excretion. Drugs may have toxic effects, either associated with the mechanism of their efficacy or due to unpredicted and unwanted activity. Candidate therapies are screened in experimental animals and in early clinical trials for such side effects, but some serious toxicities may not be recognized until wide scale utilization after drug approval.

EXAMPLES OF ANTIVIRAL THERAPY

This brief summary (see Table 16.7) provides examples of antiviral therapy to illustrate the scope and diversity of approved antiviral drugs and the variables that influence their efficacy. More detailed information is available in clinical texts and reviews.

Influenza virus

Influenza is one of the most prevalent viral diseases, affecting an estimated 10–20% of the population annually, with 3–5 million cases of severe respiratory illness and up to 500 000 deaths. In the USA alone, there is an annual excess mortality of about 50 000 attributed to influenza. As explained above, the pathogenesis of influenza – its very short incubation period of 18–72 hours and its acute course – makes it a difficult target for antiviral therapy.

Inhibitors of viral entry (M2 inhibitors)

As one step in its cellular entry pathway, influenza virus is endocytosed into an acidic vacuole; an H+ ion channel

Virus family	Specific virus and (disease)	Example of drug	Mechanism of action
Orthomyxovirus	Influenza virus (influenza)	Amantidine	Binds and blocks the H+ ion channel formed by the viral M2 proteins, prevents RNA uncoating; type A viruses only
		Oseltamivir	Binds the enzymatic site on the viral neuraminidase, prevents cleavage of terminal sialic acid residues and release of virions from infected cells; influenza A and B viruses
Retrovirus	HIV (AIDS)	Zidovudine (AZT)	Reverse transcriptase inhibitor; nucleoside analog; prevents synthesis of DNA transcripts
		Nevirapine	Reverse transcriptase inhibitor; non-nucleoside analog; prevents synthesis of DNA transcripts
		Atazanavir	Protease inhibitor; blocks processing of viral proteins
		Enfuvirtide	Entry inhibitor; oligopeptide; prevents hairpin conformational change in gp41
Hepadnavirus	Hepatitis B (hepatocellular cancer)	Entecavir	Reverse transcriptase inhibitor; nucleoside analog; prevents synthesis of DNA transcripts
Hepacivirus	Hepatitis C (chronic hepatitis)	Ribavirin	RNA mutagen, other effects?
		Pegylated interferon-α	Interferon; pleiotropic effects
Herpesvirus	Herpes simplex (encephalitis)	Acyclovir	Viral DNA polymerase inhibitor; guanine derivative; prevents synthesis of DNA transcripts
	Cytomegalovirus (retinitis)	Ganciclovir	Viral DNA polymerase inhibitor; acyclovir derivative; prevents synthesis of DNA transcripts
Poxvirus	Variola (smallpox)	Cidofovir	Viral polymerase inhibitor; cytosine derivative; prevents synthesis of DNA transcripts

TABLE 16.7 Viral diseases for which there are established antiviral drugs: some examples
After De Clercq E. Antiviral drugs in current clinical use. *Journal of Clinical Virology* 2004, 30: 115–133.

formed by the viral M2 proteins then facilitates acidification of the interior of the virion, which in turn permits dissociation of the matrix protein from the ribonucleoprotein viral core, which enters the cytosol and initiates replication. Amantadine and rimantadine, related drugs, bind in the ion channel and prevent the final step in viral entry. These drugs have been proven effective for both prophylaxis and therapy; however, drug escape mutants are frequently isolated from patients after a few days of therapy.

Inhibitors of virus release (neuraminidase inhibitors)

As influenza virus buds from a host cell, the viral hemagglutinin binds to cellular receptors, N-acetylneuraminic sialic acid residues on the cell surface. Release of free virions is accomplished by action of the neuraminidase on the viral surface, which cleaves terminal cellular sialic acid residues and frees virions to spread to adjacent uninfected cells. The neuraminidase inhibitors, oseltamivir and zanamivir, bind to the catalytic site on the neuraminidase, thereby inhibiting viral release and spread. The active domain of the neuraminidase is highly conserved in order to maintain this enzymatic function and escape mutants are relatively rare.

Not surprisingly, influenza drugs have limited efficacy in clinical application. If administered within 48 hours of the onset of symptoms, each of these drugs reduced the duration of symptoms by about 1 day and reduced the time to return to normal activity by about 1 day also. Trials have not been conducted in high risk patients with pneumonitis so there are no data on reduction in influenza mortality. In addition, these drugs are useful for prophylaxis.

Hepatitis C virus (HCV)

HCV is a hepacivirus, a positive-stranded RNA virus, in the flavivirus family. HCV has presented a difficult experimental challenge because there are limited cell culture systems and the only animal model is the chimpanzee. HCV is an important cause of human disease,

HCV genotype	Viral load before treatment	Duration of treatment (weeks)	Number of patients	Per cent patients with sustained virologic response
1	Low	24	71	52
		48	85	65
	High	24	47	26
		48	186	47
2 or 3	Low	24	47	83
		48	48	77
	High	24	97	80
		48	105	82

TABLE 16.8 A course of IFN-α 2a and ribavirin produces sustained reduction in HCV levels in serum. Patients with persistent HCV infections and evidence of chronic liver disease were randomized to several treatment regimens of 24 or 48 weeks duration. At onset of treatment, viral loads were high ($>2 \times 10^6$ HCV RNA copies per ml serum) or low ($<2 \times 10^6$ copies). After termination of treatment, there was a 12–24 weeks follow up for sustained virologic response ($<10^2$ copies). Success was influenced by HCV genotype, viral load before treatment and duration of therapy
After Hadziyannis SJ, Setter H, Morgan TR *et al.* Peginterferon-α 2a and ribavirin combination therapy in chronic hepatitis C. *Annals of Internal Medicine* 2004, 140: 346–355, with permission.

Categories	Descriptors			Treatment indicated	Success	
	HBV DNA copies per ml serum	AAT	Biopsy score		End of treatment	Sustained after treatment
Inactive disease	$<10^5$	Normal	<3	No		
Active disease HBeAg+	$>10^5$	Elevated	>4	Yes	>30%	>90%
Active disease HBeAg−	$>10^5$	Elevated	>4	Yes	~50%	>25%

TABLE 16.9 Treatment of persistent HBV infection
HBeAg is a protein encoded by HBV that is a predictor of the course of HBV disease; AAT: alanine aminotransferase level in serum, a chemical indicator of active hepatitis; biopsy score: liver biopsy, degree of necro-inflammatory changes; success: based on reduction in HBV DNA, reduction in HBeAg or HBsAg in blood and/or reduction in liver biopsy score (rates vary in different studies); sustained: at 6–12 months after ending treatment. After Lau Dt-Y, Membreno FE. Antiviral therapy for treatment-naive hepatitis B virus patients. *Gastroenterology Clinics of North America* 2004, 33: 581–599; Aggarwal R, Ranjan P. Preventing and treating hepatitis B infection. *British Medical Journal* 2004, 329: 1080–1086.

since it is estimated that there are 170 million persistently infected humans worldwide, many of whom suffer from chronic hepatitis, liver failure, or hepatocellular cancer.

The natural history of HCV is clouded by the insidious nature of infection, but it appears that 75% of initial infections are asymptomatic and ~25% of patients eliminate the virus within 3–24 months after infection. Of persistently infected patients, about 50% control viral replication so that they experience little chemical or clinical evidence of disease, while 50% have chronic hepatitis that can progress to end stage cirrhosis or hepatocellular cancer over a period of 5–40 years. These data indicate that, in many patients, there is a delicate balance between HCV and host defenses, which suggests that antiviral therapy might lead to viral clearance.

Treatment of HCV focuses on the ability to reduce or eliminate HCV in patients with persistent infections and evidence of chronic hepatitis. In a substantial proportion of patients, a combination of ribavirin and pegylated (polyethylene-glycol conjugated) IFN-α2 produces a virological response that is sustained for many months after ending a course of treatment (Table 16.8). Intensive drug discovery programs, primarily targeted to the HCV polymerase and protease, are generating promising drug candidates that have entered early clinical trials.

Hepatitis B virus (HBV)

As mentioned above, the course of HBV infection is variable and a high proportion of patients spontaneously clear the virus (see Table 16.5). Persistent HBV infections can be divided into several categories and patients with lower virus titers ($<10^5$ HBV DNA copies per ml serum) and normal liver function tests (serum alanine aminotransferase) are not usually treated since they are at relatively low risk of end stage liver disease (cirrhosis). Currently (2006), there are two treatments, either IFN or nucleoside or nucleoside phosphonate reverse transcriptase inhibitors, which are used for patients with more severe disease (Table 16.9). IFN-α administered parenterally for

Year approved	Generic name	Manufacturer
NRTIs (nucleoside reverse transcriptase inhibitors)		
1987	Zidovudine (AZT)	GSK
1991	Didanosine (ddI)	BMS
1992	Zalcitibine (ddC)	Roche
1994	Stavudine (d4T)	BMS
1995	Lamivudine (3TC)	GSK
1997	3TC + AZT	GSK
1998	Abacavir	GSK
2000	Abacavir + 3TC + AZT	GSK
2000	Didanosine	BMS
2001	Tenofovir	Gilead
2003	Emtricitabine (FTC)	Gilead
2004	Abacavir + 3TC	GSK
2004	FTC + Tenofovir	Gilead
NNRTIs (non-nucleoside reverse transcriptase inhibitors)		
1996	Nevirapine	Boehringer Ingelheim
1997	Delavirdine (DLV)	Pfizer
1998	Efavirenz	BMS, Merck
PIs (protease inhibitors)		
1995	Saquinavir	Roche
1996	Ritonavir	Abbott
1996	Indinavir (IDV)	Merck
1997	Nelfinavir	Pfizer
1997	Saquinavir Mesylate*	Roche
1999	Amprenavir*	GSK
2000	Lopinavir and Ritonavir	Abbott
2003	Atazanavir	BMS
2003	Fosamprenavir	GSK
2005	Timpranavir	Boehringer Ingelheim
Entry inhibitor		
2003	Enfuvirtide	Roche
2006	Darunavir	Tibotec

TABLE 16.10 HIV drugs approved as of 2006
Manufacturer: GSK, GlaxoSmithKline; BMS: Bristol-Myers Squibb. Saquinavir mesylate and amprenavir were withdrawn in 2005. Adapted and updated from Pommier Y, Johnson AA, Marchand C. Integrase inhibitors to treat HIV/AIDS. *Nature Reviews Drug Discovery* 2005, 4: 236–248, with permission.

months to years induces a therapeutic response in about one-third of patients (reduction in HBV DNA in serum; improved liver function tests; reduced pathology grade on liver biopsy; conversion from HBeAg+ to anti-HBeAg). Five reverse transcriptase inhibitors (RTIs) are also used, lamivudine (3TC), emtricitabine (FTC), entecavir, tenofovir or adefovir. The course of treatment is months to years and about half the patients show a therapeutic

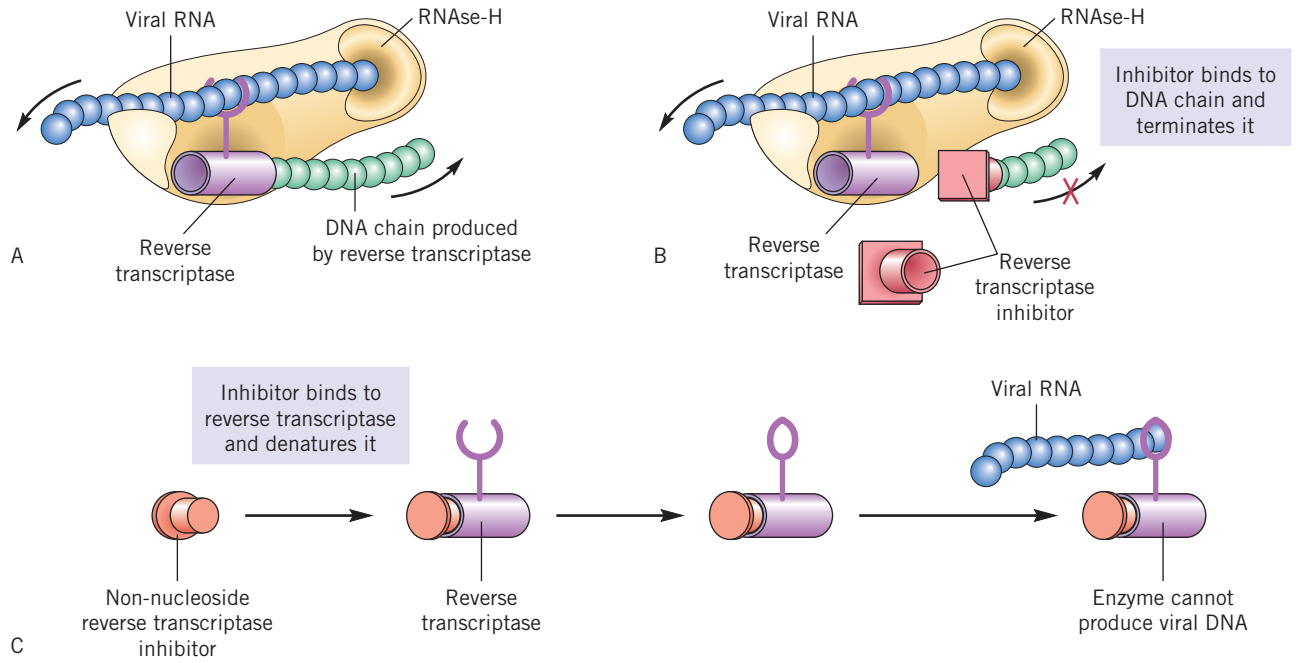

FIGURE 16.4 Mechanism of action of HIV reverse transcriptase inhibitors (RTIs). (A) Normal function of the reverse transcriptase complex. (B) Nucleoside (N) RTIs are incorporated in the DNA chain and block attachment of further nucleotides, terminating DNA synthesis. (C) Non-nucleoside (NN) RTIs bind the reverse transcriptase protein, restrict its mobility and prevent efficient transcription of DNA. After Richman DD. HIV chemotherapy. *Nature* 2001, 410: 995–1001.

response, which may last for years after end of treatment. There is no evidence that combined therapy gives a better response than monotherapy, although very high rates of resistance with lamivudine monotherapy point out the need for more effective regimens.

Human immunodeficiency virus (HIV)

There has been a greater research investment in antiviral drugs for HIV than for any other virus, because the large number of infected persons, the persistent nature of infection and the 100% fatality rate among untreated patients have created an ethical imperative and offered a very lucrative market for effective therapies. As a result, there is now a panoply of FDA-approved products (Table 16.10).

Reverse transcriptase inhibitors (RTIs)

Compounds that inhibit reverse transcription were the first drugs developed for HIV treatment. There are two types of RTIs, nucleoside analogs that bind to the nascent DNA chain and block its elongation and non-nucleoside RTIs that bind the reverse transcriptase protein and inhibit its ability to function efficiently (Figure 16.4). Drugs within each class of RTIs are not necessarily equivalent in that different HIV mutations may confer resistance to different compounds; this is important since two drugs in the same class will potentially synergize each other if they select for different resistant mutations.

Protease, entry and integrase inhibitors

The second category of HIV drugs inhibits the viral protease, which cleaves the gag polyprotein into individual functional proteins (see Chapter 14). Protease inhibitors are usually included in multidrug highly active antiretroviral regimens since they target a different function than the RTIs. More recently, enfuvirtide, the first compound that interferes with viral entry, has been approved (see discussion earlier in this chapter). The next class of HIV drugs will likely be CCR5 inhibitors or integrase inhibitors, compounds that interfere with the integration of HIV DNA transcripts into host cellular DNA.

Persistence of HIV genomes

When HIV-infected patients are started on potent combination therapy, the titers of viral RNA in plasma drop in a triphasic manner: an early and rapid decline lasting about 1 week; a slower decline lasting several weeks; and a plateau which is often below the limits of detection (see Figure 14.11). However, if treatment is stopped, viremia inevitably reappears, usually in a few weeks, indicating that viral eradication has not been achieved. Persistence is likely due to several phenomena, including latent viral genomes in resting CD4+ T cells and continuing low level replication, both in sequestered sites such as the brain and genital tract, and in resting T cells (see Chapter 14). For these reasons, it appears unlikely that drug treatment, even with future advances, will lead to a 'cure' of this infection.

Herpesviruses

Viral polymerase inhibitors

Herpesviruses are large DNA viruses that encode their own DNA polymerases, which are required for transcription of their genomes. Acyclovir, the compound usually preferred for treatment of herpes simplex and

varicella zoster viruses, is a DNA chain terminator, which resembles guanosine but has a sidechain that lacks the ribose ring. Acyclovir undergoes in vivo activation by addition of three phosphates to its sidechain. The first phosphate is added by a virus-encoded thymidine kinase so that the drug can only act in HSV- and VZV-infected cells, while the second and third phosphate are added by cellular kinases. Acyclovir triphosphate is incorporated into nascent DNA chains but, absent a ribose, the DNA polymerase cannot add further nucleotides and the chain is terminated. Valacyclovir is the valine derivative of acyclovir, a prodrug of acyclovir that is more bioavailable upon oral administration. Famciclovir is another widely used nucleoside with similar selectivity for the thymidine kinase and polymerase of HSV and VZV. Ganciclovir is a derivative of acyclovir that can be phosphorylated by the protein kinase of cytomegalovirus (CMV) and is used for treatment of CMV retinitis and other complications of AIDS and transplantation.

REPRISE

Molecular studies of viral infection have identified potential targets for antiviral therapeutics, which include viral proteins and nucleic acids, as well as selected cellular proteins. In addition, enhancement of natural host defenses, both innate and acquired, may be exploited for therapeutic purposes. Pathogenesis of different viral infections also contributes to identification of the best disease targets for chemotherapy, particularly persistent infections that are widespread and carry high risk of morbidity and mortality. Using this information, pharmaceutical companies have the research tools that enable them to develop or select compounds that have significant antiviral activity. However, in vivo bioavailability, pharmacodynamics and potential toxicity are additional major hurdles that must be overcome during the development of safe and effective antiviral chemotherapeutics.

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Chapter 17

Viral Vaccines

Neal Nathanson and Harriet L. Robinson

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REPRISE

ACKNOWLEDGEMENT

FURTHER READING

VIRAL PATHOGENESIS AND VACCINE-INDUCED PROTECTION

The prevention of viral diseases by the use of vaccines has been one of the great successes of preventive medicine. Jenner's introduction of vaccinia immunization to ameliorate smallpox is an 18th century landmark in public health and one of the earliest demonstrations of the basic principles of immunology. Vaccinia also provided a precedent for the use of live attenuated viruses to induce effective long-lasting protection, an example that even today inspires vaccinologists. During the last half of the 20th century a large number of safe and effective viral vaccines were developed for use in humans and animals. However, vaccine development has been largely an empirical science and there is a paucity of data on the mechanisms of protection.

This chapter is based on the premise that vaccine-induced protection can best be understood in the context of viral pathogenesis, which identifies potential steps in the infectious process where immunity might intervene to prevent disease. The major vaccine modalities are described, with their strengths and limitations, followed by an analysis of the mechanisms of vaccine-induced protection as exemplified by a few of the best studied vaccines. Finally, progress in development of an AIDS vaccine is summarized to illustrate one of the unsolved challenges in vaccinology.

VACCINE MODALITIES

There are certain immunological principles that govern the induction of protective responses by any vaccine modality (Sidebar 17.1). Delivery of an immunogen to professional antigen presenting cells (APCs) is the most effective way to initiate immune induction, which can be modulated to emphasize either cellular or humoral responses. There is a physiological limit to the expansion of naïve T lymphocytes during the primary response but, once rested, committed memory lymphocytes can be re-stimulated to undergo further expansion (often called an anamnestic or booster response). Adjuvants can bring professional APCs into contact with antigens through their pro-inflammatory action or exploit cytokines to increase proliferation of antigen-responsive lymphocytes. Newer vaccine modalities attempt to exploit these immunological principles both to enhance and focus the immune response to maximize protective efficacy.

Vaccine modalities fall into three broad categories: attenuated live viruses; non-replicating purified antigens; and vectors with limited replicative

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SIDEBAR 17.1

Principles of immune induction relevant for vaccine efficacy

- Immune induction is much more efficient if an immunogen is presented by professional APCs, such as macrophages and dendritic cells.
- For many viral infections, both immunoglobulin and cellular effector systems can participate in protective immunity, but their relative role varies for different viruses.
- Immune induction can be manipulated to favor either T_H1 (cellular) or T_H2 (antibody) responses, by formulation of immunogen, route of immunization, and the use of adjuvants.
- Adjuvants can enhance the immune response in a variety of ways, mediated by their induction of pro-inflammatory cytokines.
- Presentation of antigen to the mucosa-associated lymphoid system (MALT) can induce local immunity, which may provide an effective barrier to viruses that invade via mucosal tissues.

capacity. Each of these modalities has its advantages and disadvantages and it is unpredictable which one will produce the most successful vaccine for a given viral disease (Table 17.1). The earliest vaccines were attenuated viruses that were derived using primitive methods, although in some instances molecular sequencing and virus cloning have been employed to produce improved versions. With the beginnings of experimental virology, technology was developed that led to the earliest non-replicating viral vaccines, formulated by chemical or physical inactivation of virulent viruses. Further advances permitted the production of recombinant viral proteins that could be used as immunogens. Most recently, a variety of vector systems has been introduced to express viral proteins and these are currently under active development as potential vaccine modalities.

Table 17.2 contains a list of the viral vaccines in common use for humans. Most of these are either live attenuated viruses or inactivated viruses, since the newer vector systems are yet to be adopted as standard approved vaccines.

Safety and efficacy	Advantages and disadvantages
Live attenuated viruses	
Safety	*Advantages* None
	Disadvantages Residual pathogenicity Reversion to increased pathogenicity Unrecognized adventitious agents Possible persistence
Efficacy	*Advantages* Local immunity at portal of entry Cellular and humoral immunity induction Long-lasting immune response Herd immunity Less expensive to manufacture
	Disadvantages Interference between serotypes Interference by adventitious viruses Loss of infectivity on storage Cold chain required to maintain infectivity
Inactivated or subunit viruses, or recombinant proteins	
Safety	*Advantages* Avoids dangers of attenuated viruses
	Disadvantages Potential residual infectious pathogenic virus Safety tests difficult and expensive Induction of unbalanced immune response
Efficacy	*Advantages* No viral interference Avoids limitations of attenuated viruses
	Disadvantages No induction of local immunity Poor induction of cellular immunity May not mimic native epitopes for humoral immunity Short duration immunity (some products) More expensive to manufacture

TABLE 17.1 The advantages and disadvantages of different vaccine modalities: attenuated viruses and non-replicating antigens

Attenuated viruses

Attenuated viruses produce infections that are milder than the illnesses produced by the counterpart virulent wildtype viruses from which they are derived. Attenuated variants may differ in several ways from wildtype isolates (see Chapter 9). They are often host range mutants so that their replicative capacity, relative to their wildtype counterparts, is high in selected cell culture systems but much lower in vivo. Also, attenuated vaccine viruses are selected for differential tropism in vivo compared to their virulent parents. For instance, cold adapted influenza viruses will replicate quite well at 33°C but poorly at 37°C (see Table 9.5). In vivo, the cold adapted virus replicates in the upper respiratory tract (nasal epithelium) but very little in the lower respiratory tract (alveolar epithelium), while the virulent virus replicates well in both sites. Attenuated oral poliovirus vaccine (OPV) exhibits a different pattern of tropism than does wildtype poliovirus, since it replicates well in the gastrointestinal tract but poorly in the central nervous system (CNS), while wildtype virus replicates robustly in both sites (Table 17.3). Also, OPV causes little or no viremia so it rarely reaches

Date of approval USA	Virus and disease	Vaccine modality Route administration	Currently used in USA?
Before 1900	Variola Smallpox	Attenuated Intradermal	Only in the event of exposure
~1939	Yellow fever	Attenuated Subcutaneous	Only in the event of exposure
1955	Polio Poliomyelitis	Inactivated Intramuscular	Yes, all infants
1963	Polio Poliomyelitis	Attenuated Oral	Yes, special circumstances
1963	Measles	Attenuated Subcutaneous	Yes, all infants
1967	Mumps	Attenuated Subcutaneous	Yes, all infants
1969	Rubella German measles	Attenuated Subcutaneous	Yes, all infants
1971	Influenza	Inactivated Intramuscular	Yes, high risk only
1980	Rabies	Inactivated Intramuscular	Yes, high risk only
1981	Hepatitis B	Inactivated Intramuscular	No, no longer made
1986	Hepatitis B	Recombinant HBs protein Intramuscular	Yes, all infants
1995	Varicella Chicken pox	Attenuated Subcutaneous	Yes, all infants
~1996	Hepatitis A	Inactivated virus Intramuscular	Yes, high risk only
2006	Rotavirus Infant diarrhea	Attenuated Oral	Yes, infants
2006	Papillomavirus Cervical carcinoma	Recombinant L1 protein Intramuscular	Adolescents?

TABLE 17.2 Approved human viral vaccines, a selected list (2006)
For definitive information see Plotkin SA, Orenstein WA (eds). *Vaccines*, 4th edn, Saunders, Philadelphia, 2004.

the CNS. These properties reduce the pathogenicity of the attenuated virus while retaining its immunogenicity.

Efficacy

In general, infection with a wildtype pathogenic virus induces long-lasting, often lifelong, immunity that protects against illness upon re-exposure to the same virus. Therefore, the immunity induced by 'natural' infection has been considered the 'gold standard' for vaccines. Because attenuated variant viruses can, under optimal circumstances, mimic the protection afforded by natural infection, they are often considered the vaccine modality of choice.

Attenuated viruses are usually effective immunogens, assuming that they replicate sufficiently. The full cycle of replication in vivo, regardless of the specific cellular target, appears to provide sufficient protein substrate to professional APCs to load both class I and class II MHC molecules with oligopeptides, in addition to binding immunoglobulin molecules to stimulate cognate B lymphocytes (see Chapter 6). Laboratory passage and cloning of viruses can produce variants with different degrees of attenuation as measured by the mildness of illness and the reduction in in vivo replication and the degree of attenuation usually reflects the number of attenuating mutations. In general, immunogenicity decreases with reduction in in vivo replication and 'over-attenuation' can lead to viruses that are poorly immunogenic.

The advantages and disadvantages of attenuated viruses as efficacious vaccines arise from the requirement

Type 1 poliovirus strain	TCD50 per ml	Enterotropism TCD50 per po ID50	Neurotropism TCD50 per ic PD50
Virulent Mahoney (CNS suspension)	10^6	$10^{3.3}$ (monkeys)	$10^{1.9}$
Attenuated LSc (tissue culture fluid)	$10^{7.6}$	$\sim 10^4$ (humans)	$>10^{7.6}$

TABLE 17.3 Attenuated vaccine viruses often exhibit a different pattern of tropism than the corresponding wildtype viruses. A virulent and attenuated type 1 poliovirus are compared to show that they both replicate well in cell culture (primary monkey kidney cells) and are enterotropic (infectious after oral (po) administration) but differ markedly in their neurovirulence after intracerebral (ic) injection in cynomolgus monkeys
PD50: 50% paralytic dose; ID50: 50% infectious dose. After Sabin AB, Hennessen WA, Winsser J. Studies on variants of poliomyelitis virus. *Journal of Experimental Medicine* 1954, 99: 551–576; Sabin AB. Properties and behavior of orally administered attenuated poliovirus vaccine. *Journal of the American Medical Association* 1957, 164: 1216–1223, with permission.

Location	OPV	Per cent seroconversion		
		Type 1	Type 2	Type 3
Toluca, Mexico Tropics	Trivalent	68	82	43
Leningrad, USSR Winter	Trivalent	82	80	71
Leningrad, USSR Winter	Monovalent Order: 1, 3, 2	97	100	96

TABLE 17.4 Some attenuated virus vaccines can be subject to interference under certain conditions. Frequency of seroconversion following administration of oral poliovirus vaccine (OPV) to children in the tropics compared to seroconversion following OPV administration during the winter months in a temperate climate. Conversions are greater in Leningrad (now St Petersburg) in the winter when other enteroviruses are relatively infrequent compared to Toluca where ~50% of children are excreting non-polio enteroviruses at any one time. Also, type 2 interferes with the other types and type 3 is most subject to interference. When the three types are fed individually and sequentially, the proportion immunized is greater than is elicited by trivalent vaccine
After Sabin AB, Alvarez MR, Amezquita JA *et al.* Effects of rapid mass immunization of a population with live, oral poliovirus vaccine under conditions of massive enteric infection with other viruses. *Second international conference on live poliovirus vaccines*, Scientific Publication 50, WHO, Geneva, 1960, with permission.

for replication in the non-immune host. For instance, oral poliovirus vaccine (OPV) is subject to 'interference' due to concomitant silent natural infection with other enteroviruses. As a result, when OPV is administered in mid winter in areas with a temperate climate, a high proportion (>90%) of seronegative vaccinees undergo immunizing infections. In contrast, a single dose of trivalent OPV can produce a lower frequency of 'takes' when administered to young children in the tropics (Table 17.4). OPV is a trivalent vaccine that includes types 1, 2 and 3 strains of virus. When OPV was under development, it was found that there was interference between the three types and the relative titers had to be carefully balanced to achieve the maximal number of conversions to all three types following oral administration to seronegative children.

Safety

The search for an acceptable attenuated vaccine strain requires identification of variants that fall in a putative window of robust immunogenicity with minimal disease potential. In spite of diligent efforts to achieve complete safety, some attenuated vaccine viruses retain residual pathogenicity. For instance, OPV causes an occasional case of paralytic poliomyelitis, at a frequency of about 2 cases per 1 000 000 primary immunizations (Table 17.5).

Virus	Study period	Paralytic rate per 10^6 primary infections or immunizations	Relative rates
Wildtype	1931–1954	7000	~3000
OPV	1961–1978	2.3	1

TABLE 17.5 Attenuated virus variants used for vaccines often retain some residual pathogenicity. In this example, the attenuated vaccine virus (OPV, oral poliovirus vaccine), that is highly attenuated but still causes a small number of cases of paralysis, is compared to wildtype poliovirus
After Nathanson N, McFadden G. Viral virulence, Chapter 5 in Nathanson N, Ahmed R, Gonzalez-Scarano F *et al.* (eds), *Viral pathogenesis*, Lippincott Raven Publishers, Philadelphia, 1997, with permission.

In addition, some attenuated vaccine viruses may revert in virulence during passage in the primary vaccine recipient, which can be a problem if the virus is excreted. Thus, OPV often increases in virulence upon a single human passage due to revertant mutations and, in the period 2000–2005, several small outbreaks of poliomyelitis were traced to revertant strains of vaccine virus.

Another problem with live virus vaccines is that they may be inadvertently contaminated with adventitious agents. For instance, yellow fever vaccine produced

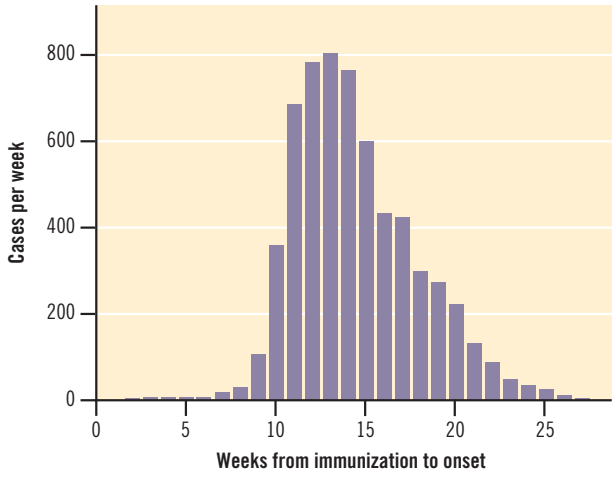

FIGURE 17.1 Live attenuated vaccine viruses may be inadvertently contaminated with extraneous infectious agents capable of causing disease. In this example, a batch of yellow fever virus was contaminated with HBV introduced with human serum used to 'stabilize' the yellow fever vaccine. Investigation showed that one of the serum donors was an asymptomatic carrier of HBV. The graph shows cases tabulated by weeks from immunization with 17D yellow fever vaccine to onset of jaundice. After Sawyer WA, Meyer KF, Eaton MD, Bauer JH, Putnam P, Schwentker FF. Jaundice in army personnel in the western region of the United States and its relation to vaccination against yellow fever. *American Journal of Hygiene* 1944, 39: 337–387.

a massive epidemic of hepatitis B in the 1940s that was traced to a batch of vaccine that contained human serum obtained from an asymptomatic individual who was later shown to be a carrier of HBV (Figure 17.1). Another contaminant of yellow fever vaccine was avian leukosis virus, acquired from the eggs used to prepared chick embryo cultures in which the vaccine virus was grown; this problem has now been eliminated by using leukosis-free eggs.

Inactivated viruses, subunit vaccines and recombinant proteins

Inactivated viruses are the other common modality among approved viral vaccines. A number of chemical and physical methods can be used to inactivate viruses without destroying the integrity of the virus particle or much of its antigenicity. For instance, inactivated poliovirus vaccine is manufactured by treating the virus with dilute formalin (formaldehyde gas dissolved in water) at 37°C for several weeks. The chemical treatment denatures the outer capsid protein sufficiently to prevent viral attachment and entry, while retaining epitopes that induce neutralizing antibodies. Beta propriolactone is another chemical that acts in a manner similar to formalin and has been used to prepare inactivated rabies virus vaccines. An alternative is a so-called 'split product' vaccine, produced by treatment of the virion with mild detergent or ethyl ether that dissociates the particle to yield a suspension of proteins and nucleic acids that are non-infectious but retain antigenicity. This method has been used to produce influenza virus vaccines.

Efficacy

Inactivated virus products are usually injected either intradermally, subcutaneously or intramuscularly. Such vaccines induce circulating antibodies, but they are usually poor at inducing mucosal (IgA) antibodies because the antigens are not presented to the mucosal associated lymphoid tissue. Also, it is thought that inactivated virus vaccines are poor inducers of cellular immune responses, although most have never been examined using modern methods to measure cellular immunity. Finally, it is often asserted that immune responses induced by inactivated vaccines are not long lasting, based on the view that continuous antigenic stimulus is required to maintain immune responses. However, in some experimental systems it appears that antigen-committed memory T cells can persist for long periods of time in the absence of antigen (see Chapter 6). Furthermore, there are examples of antibody responses induced by inactivated vaccines that appear to be long lasting by comparison with the responses induced by the cognate wildtype virus.

Safety

Inactivated virus vaccines are often formulated from pathogenic virus strains and their safety is contingent upon total inactivation. Successful inactivation requires a careful scientific analysis of the mechanism and kinetics of inactivation and the ability to test each vaccine batch for residual virus. On occasion, failures in inactivation have caused cases of disease, such as occurred during the Cutter 'incident' that confounded the introduction of inactivated poliovirus vaccine. A related problem is that 'over-inactivation' done to insure safety can compromise the immunogenicity of inactivated vaccines.

On rare occasions, inactivated vaccines can induce an 'imbalanced' immune response that leads to untoward effects. For instance, inactivated measles virus elicited an immune response that resulted in enhanced disease. When children immunized in this manner were exposed to natural measles, they were not protected but developed 'atypical' measles with unusual symptoms. Similarly, early trials of an inactivated vaccine against respiratory syncytial virus, an important respiratory virus of children, resulted in enhanced disease rather than protection.

Recombinant proteins

A modern alternative to inactivated viruses is the preparation of a recombinant viral protein for use as an immunogen. A successful example is the use of HBsAg (hepatitis B surface antigen), the envelope protein of HBV, as a vaccine. The original HBV vaccine was prepared by purifying the virus from the serum of human carriers, since it was impossible to grow HBV in cell culture. The harvested virus was purified and inactivated with formalin. However, this process was fraught with dangers, such as the incomplete inactivation of HBV or the possibility of accidentally including extraneous agents present in donor serum. In this instance, the recombinant protein was clearly a safer product and luckily it

proved to be very effective as a vaccine. However, industrial scale production, purification and stabilization of recombinant proteins is a daunting challenge and such products are often expensive to manufacture.

Vectors: recombinant viruses, replicons and DNA vaccines

In the last few years, there has been a burst of research activity dedicated to novel modes of antigen presentation, sometimes called vectors or 'platforms'. These new approaches include recombinant viruses, replicons and purified DNA.

Recombinant viruses

DNA viruses are readily engineered to introduce new genetic sequences, which can also be done with RNA viruses whose genomes can be transcribed into infectious DNA clones. Although many virus genomes can be manipulated to express foreign antigens, the largest viruses, such as poxviruses and herpesviruses, are most suitable for this purpose. Poxviruses have been used more frequently than other viruses and vaccinia virus is the basis for some licensed animal vaccines, such as a rabies virus vaccine that has been deployed for the successful immunization of wildlife.

There are distinct limits to the amount of genetic information that can be added to the genome of smaller viruses without compromising their replicative capacity. However, smaller genomes may be used successfully in specialized instances. For instance, the 17D attenuated vaccine variant of yellow fever virus can be chimerized to express the surface glycoprotein of other virulent flaviviruses, such as Japanese encephalitis or West Nile viruses, providing an immunogen with established safety that will induce neutralizing antibodies against a human pathogen. Likewise, dengue virus, another flavivirus, can be chimerized with Langat virus to produce an attenuated recombinant virus that induces protective immunity against tick-borne encephalitis viruses.

There are several considerations in selecting a replicating virus for use as a vaccine 'platform', including safety, immunogenicity and prior immunity of the target population. Current safety standards make it much more acceptable to use a virus that has already had widespread use in the human populations, such as vaccinia virus. Even here there are safety problems, since vaccinia causes serious complications, albeit at the frequency of <1 case per 100 000 vaccinees. Thus, certain attenuated strains of vaccinia virus, such as MVA (modified virus Ankara) or NYVAC are preferred to standard vaccinia virus.

The immunogenicity of a recombinant virus depends in part on the cells that it targets. Some viruses infect macrophages and dendritic cells and this maximizes their ability to deliver proteins to professional APCs, thereby enhancing the immunogenicity of the recombinant proteins that they encode. Since many recombinant constructs are based on human viruses, vaccinees may have been previously infected with the wildtype counterpart and this pre-existing immunity can reduce the replication of the recombinant virus and compromise its immunogenicity. For instance, recombinant vaccinia viruses are somewhat less immunogenic in persons who were previously vaccinated than in vaccinia-naïve subjects. Recombinant adenoviruses have proven to be highly immunogenic vectors but are less effective in subjects already immune to the serotype used in the vaccine construct.

Replicons

Replicons are virus-like particles that will enter a target cell, undergo limited transcription and translation to synthesize encoded proteins, but will not produce infectious progeny. Replicons consist of a virus genome that has been engineered to insert a new protein and to delete some of the genes of the parent virus. Such genomic constructs are often transfected into packaging cell lines that provide a viral envelope in trans, permitting the assembly of a virus-like particle with the cellular specificity associated with the envelope (Figure 11.8). In contrast to recombinant viruses, replicons cannot spread beyond the cells that they initially 'infect'. Replicons are a lower risk platform than recombinant viruses and can exploit the attributes of many wildtype viruses that would be unacceptable for use as an infectious recombinant virus.

The efficacy of replicons depends upon their ability to reach a sufficient number of target cells, to produce enough novel immunogen and to deliver the immunogen to professional APCs. In addition, it may be difficult to produce certain replicons on the industrial scale needed for vaccine deployment. Finally, replicons must pass safety tests based on the assurance that they will not recombine with cellular sequences to reconstitute the potentially pathogenic viruses from which they are derived. Only future investigation will determine whether replicons are a practical platform for vaccine formulation.

DNA-based immunogens

It was first discovered in the early 1990s that a DNA plasmid, encoding a protein, could be used as an immunogen by simple injection of the 'naked' DNA. This novel technology is currently under active investigation. DNA vaccine plasmids usually utilize a promoter, such as the CMV (cytomegalovirus) promoter, that is highly active in most eukaryotic cells, driving a genetic insert expressing the protein of interest, followed by a transcriptional terminator and a polyadenylation sequence. Modifications of the protein sequence, such as a signal sequence or a transmembrane domain, can be used to influence how the protein is processed in APCs.

DNA constructs are usually administered intramuscularly using a hypodermic needle or into the epidermis using a gene gun, which bombards the skin with gold beads coated with DNA. To be immunogenic, the DNA-encoded protein must be presented by professional APCs. Proteins expressed in epithelial cells would be taken up by APCs via the exogenous pathway, while proteins

expressed in APCs could enter the endogenous pathway. Gene gun injections induce responses with less DNA than is required for soluble DNA, but tend to induce T_H2 responses biased toward antibody. DNA immunogens may be enhanced by the use of adjuvants. For instance, unmethylated CpG motifs in plasmid DNA provide a T_H1-biased adjuvant effect through toll-like receptors. Also, DNA can be adjuvanted with plasmids encoding cytokines such as IL-2. DNA-based immunogens, used for single or multiple injections, have shown modest immunogenicity, but have been more effective when used in concert with other vaccine platforms. DNA-based immunogens are currently used mainly to prime an immune response followed by boosting with another vaccine modality, a type of vaccination called heterologous prime/boost (see Figure 17.13 below).

As a vaccine, DNA possesses several advantages. First, it represents a well-defined and stable immunogen that can be precisely characterized and controlled and produced on a large scale at relatively low cost. It appears to be biologically safe, assuming that it is adequately purified and it avoids some of the dangers intrinsic in attenuated viruses, inactivated viruses and certain vectors. Also, DNA is not subject to pre-existing immunity, a phenomenon that limits the effectiveness of live recombinant viruses to which some humans are already immune, because of prior infection with their wildtype counterparts or because of their use in other vaccines.

Adjuvants

Sometimes called 'the immunologist's dirty little secret', adjuvants have long been known to enhance the immunogenicity of antigens, particularly foreign proteins. The classic adjuvant is Freund's complete adjuvant (FCA), an oil-water emulsion containing tubercle bacillus and the selected foreign protein. However, FCA caused granulomas at the site of injection and is not acceptable for use in humans. Aluminum oxides (alum) are much less irritating and are used in some human vaccines. Recent understanding of the innate immune system (see Chapter 5) has illuminated the mechanisms by which adjuvants appear to operate. Most of them bind to one or more of the toll-like receptors (TLRs), thereby activating dendritic cells and increasing the production of pro-inflammatory cytokines, as well as drawing macrophages to the site of antigen deposition. This amplifies the amount of the antigen that is bound by professional APCs and increases the number of antigen-specific T cells that respond to the antigen.

SIDEBAR 17.2

Mechanisms of vaccine-induced protection against viral diseases – some tentative principles

- The mode of vaccine-induced protection can be best understood in the context of the pathogenesis of a specific viral infection.
- Immune mechanisms involved in pre-exposure protection are different than those involved in recovery from primary infection.
- Vaccine-induced protection may be due to a combination of several protective mechanisms rather than a single component of the immune response. Different mechanisms may be involved in protection against different viruses.
- Neutralizing antibody, if it is present at the portal of entry and at the time of infection, can act more rapidly than any other defense to inactivate the challenge virus.
- Cellular immune responses, mediated by a variety of cell types and acting through diverse mechanisms, may contribute to protection, particularly if the challenge virus is not 'sterilized' at the portal of entry.

MECHANISMS OF PROTECTION BY ESTABLISHED VACCINES

A large number of viral vaccines have been developed, licensed and are in use for the prevention of disease in humans (see Table 17.2). These successful established products provide a potential source of information about the ways in which a vaccine confers protection. To elucidate these mechanisms, information on several of these vaccines is reviewed below. Since most relevant research was conducted decades ago, prior to the introduction of modern methods for the measurement of cellular immune responses, the data focus mainly on the role of antibody in vaccine-induced protection. Keeping this caveat in mind, a few generalizations can be made about the mechanism of vaccine-induced protection (Sidebar 17.2).

Poliovirus

The pathogenesis of poliovirus is understood at an organ level, although many of the specific cellular details have never been elucidated. As shown in Figure 17.2, the virus is ingested and invades via the tonsils and the lymphoid tissue of the small intestine, spreads to regional lymph nodes and is transmitted through efferent lymphatics into the blood, where it circulates as a cell-free plasma viremia. Blood-borne virus invades the central nervous system either directly across the blood–brain barrier or indirectly by invading peripheral nerves or peripheral ganglia followed by neuronal spread to the CNS. After injecting a virulent wild type virus into macaques (see Figure 2.5), viremia is observed for about one week, followed by the appearance of neutralizing antibody, simultaneous with the disappearance of infectious virus. These observations suggest that a potential weak link in the pathogenesis is the transit of virus through the blood and that antibody might be capable of blocking that step and preventing invasion of the target organ.

To test the hypothesis that neutralizing antibody might protect against the paralytic consequences of poliovirus infection, experiments can be performed with passive immunization. Monkeys are injected with graded doses of a pool of anti-poliovirus antiserum.

One day after injection, the titer of passive antibody in the serum of the recipient animals is measured and the animals are challenged with a dose of virulent poliovirus. The results provide clear evidence that antibody, present prior to virus challenge, protects against paralysis (Figure 17.3). This strongly suggests that a vaccine that elicited neutralizing antibody might protect against paralytic poliomyelitis.

These considerations led to the formulation, by Jonas Salk, of an inactivated preparation of poliovirus (IPV) as a candidate immunogen. The 1954 Field trial of IPV provided an opportunity to test the hypothesis that neutralizing antibody could account for protection. The proportion of vaccinees, seronegative prior to immunization, who responded with different levels of antibody, was compared with the estimated efficacy of the vaccine (Table 17.6). There was a good correlation between the proportion of vaccinees who responded at a titer of 1:4 or greater and the estimated efficacy of the vaccine (~65%). This correlation suggested that a minimal level of neutralizing antibody could account for protection.

When attenuated strains of poliovirus were licensed as an oral poliovirus vaccine (OPV), it became possible to compare the ability of IPV and OPV to prevent enteric infection, a different aspect of vaccine-induced protection. Such a comparison indicated that IPV conferred minimal protection against enteric infection, but that OPV reduced fecal excretion significantly (Figure 17.4). The ability of OPV to induce mucosal immunity was confirmed by demonstrating low levels of anti-poliovirus IgA in fecal samples from subjects immunized with OPV. It seems likely that OPV generates local immunity by inducing antibody production by B cells in the gastrointestinal-associated lymphoid tissue (GALT), although there is little direct evidence for this speculation.

The widespread use of poliovirus vaccines has led to the eradication of wildtype polioviruses from the USA (about 1980) and from the rest of the Western hemisphere (about 1995). By any standard, this is an impressive success. Can this efficacy be attributed entirely to

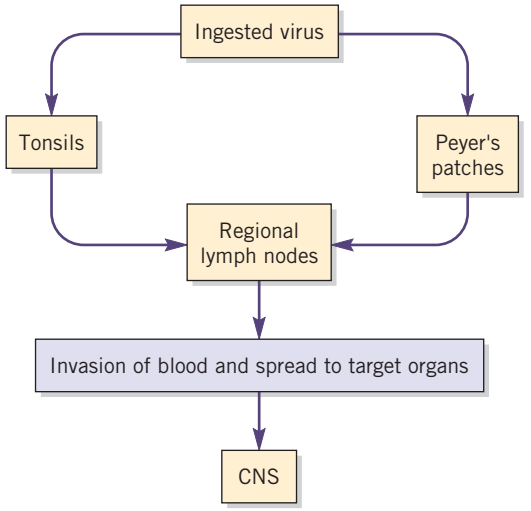

FIGURE 17.2 Pathogenesis of poliovirus infection. This diagrammatic summary indicates that the virus invades via tonsils and Peyer's patches (lymphoid tissue accumulations in the walls of the small intestine), spreads to regional lymph nodes, produces a plasma viremia and then invades the central nervous system. A variant of this scheme, not shown, suggests that the virus invades the peripheral nervous system from the blood and travels thence to the CNS. After Bodian D. Emerging concept of poliomyelitis infection. *Science* 1955, 122: 105–108, with permission.

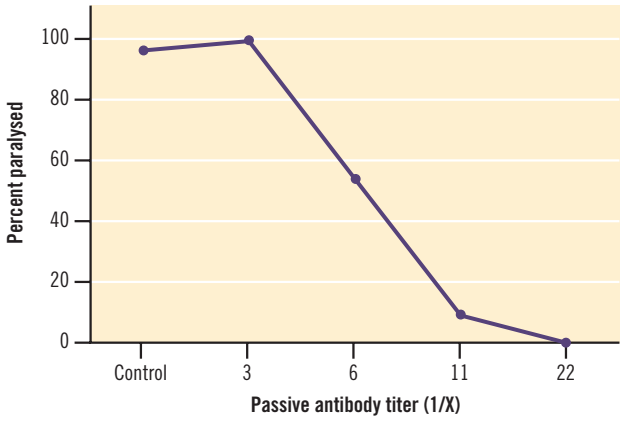

FIGURE 17.3 Passive antibody protects against paralytic poliomyelitis. In this experiment, cynomolgus macaques were given a single intramuscular injection of graded doses of poliovirus immune immunoglobulin. One day later the level of neutralizing antibody was determined and the animals were challenged with the virulent Mahoney strain of type 1 poliovirus by the intramuscular route. An antibody titer of ~1:10 conferred >90% protection. After Nathanson N, Bodian D. Experimental poliomyelitis following intramuscular virus infection. III. The effect of passive antibody on paralysis and viremia. *Bulletin of the Johns Hopkins Hospital* 1962, 111: 198–220, with permission.

	Unvaccinated placebo	Vaccinated	Per cent protection against paralytic poliomyelitis
Number of paralytic cases	40	14	65
Per cent seroconversions after vaccination (⩾1:4)	0	65	

TABLE 17.6 A low level of serum neutralizing antibody correlates with protection against paralytic poliomyelitis. In 1954, a large scale clinical trial was done to test whether a poliovirus vaccine (IPV) would protect against poliomyelitis. An equal number of children were assigned to the vaccinated and unvaccinated placebo groups (about 200 000 in each group) so the numerators can be directly compared; paralytic cases were reduced by 65% in the vaccinated group ((40–14)/40 = 65). To determine the level of antibody that correlated with protection, the proportion of vaccinated children with seroconversions at different titers were compared with the proportion protected by the vaccine. Of the children who were seronegative prior to immunization, ~65% had a titer of ⩾1:4 after the full course of three doses of vaccine. Since this corresponded to the per cent who were protected against paralytic poliomyelitis, it was concluded that a titer of ⩾1:4 probably was sufficient to protect against paralytic disease. This analysis is limited to type 1 poliovirus, which was responsible for about 80% of all cases of paralytic poliomyelitis
After Francis TJ Jr, Napier JA, Voight R *et al. Evaluation of the 1954 field trial of poliomyelitis vaccine.* School of Public Health, University of Michigan, Ann Arbor, Michigan, 1957, with permission.

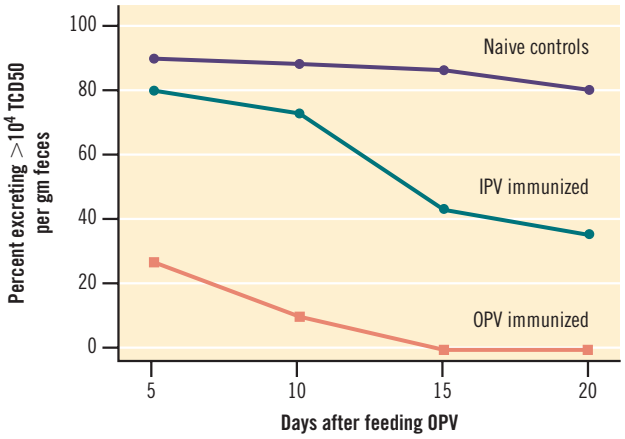

FIGURE 17.4 OPV (oral poliovirus vaccine) provides greater protection against enteric infection than does IPV (inactivated poliovirus vaccine). Three groups of children (naïve unimmunized; immunized with OPV; immunized with IPV) were tested for fecal excretion after feeding of OPV. Since the serum antibody levels were similar in the OPV and the IPV immunized groups, it was inferred that OPV induced local mucosal immunity more effectively than did IPV. After Henry JL, Jaikara ES, Davies JR *et al*. A study of poliovaccination in infancy: excretion following challenge with live virus by children given killed or living poliovaccine. *Journal of Hygiene* 1966, 64: 105–120, with permission.

neutralizing antibody? Information relevant to this question has been derived from the cumulative experience with OPV over the last 40 years. It was noted above that OPV causes rare cases of poliomyelitis in vaccine recipients (about 2 per million in vaccine recipients). Some of these cases are in children with inherited immunodeficiencies that were unrecognized at the age (1–6 months) when they were immunized. Uniformly, these children have been diagnosed as hypo- or agammaglobulinemic. Strikingly, children with inherited T cell defects (such as the di George syndrome) do not seem to be at risk of vaccine-associated poliomyelitis. Furthermore, some of these children with immunodeficiency-associated poliomyelitis continue to excrete the vaccine virus for months to years, in spite of the fact that many of them are treated with pooled normal immune globulin (by intramuscular injection).

These observations imply that clearance of enteric poliovirus replication is mediated by antibodies. Furthermore, the failure of systemic globulin treatment (which would provide circulating neutralizing poliovirus antibodies) to clear infection suggests that antibody produced by the GALT is important for the clearance of enteric infection. The absence of any data on the development of cellular immune responses to poliovirus vaccines precludes definitive conclusions, but there is little suggestion that CD8-mediated mechanisms play a role in protective immunity against poliovirus.

Rabies virus

Rabies presents a special challenge for immunization, in part because of its unusual pathogenesis and, in part, because it is one of the few infections where post-exposure vaccination is frequently used. Rabies virus is often acquired through the bite of a rabid animal (see Figure 1.1). Following injection into muscle or other peripheral site, the virus replicates locally, crosses the neuromuscular junction and travels by the neural route to the CNS where it produces a fatal encephalomyelitis (Figure 2.2). Importantly, rabies virus never produces a viremia.

One peculiar aspect of rabies pathogenesis is the variability in the incubation period. The virus may transit to the CNS within a few days or may be sequestered in an extraneural site for weeks to months before it invades the nervous system. The length of the rabies incubation period is determined by a variety of parameters, particularly the strain of virus. Thus, a neuro-adapted rabies virus, CVS (challenge virus standard), produces rabies with a high frequency and a short incubation period, while a freshly isolated wildtype strain (so-called 'street' virus) usually produces a lower frequency of 'takes' and a much longer incubation period. Other parameters that influence incubation period are virus dose and experimental host.

The long incubation period following exposure to street rabies virus provides the opportunity for post-exposure prophylaxis. In the USA, pre-exposure vaccination is limited to veterinarians or others who are at occupational risk. Because the general population is not routinely immunized, post-exposure prophylaxis is the major mode of rabies prevention. The protective mechanisms of pre- and post-exposure propylaxis are probably somewhat different and are considered separately.

Pre-exposure prophylaxis

It appears that neutralizing antibody plays an important role in pre-exposure prophylaxis. Passive administration of antibody will protect animals against subsequent challenge with rabies virus, the degree of protection being correlated with the titer of antibody, the timing of administration and the strain and dose of rabies virus used for infection. If a group of animals is immunized with a rabies vaccine and tested for antibody just before challenge by injecting rabies virus at a peripheral site, there is a strong correlation between antibody titer and the degree of protection (Table 17.7). Further evidence for the protective role of antibodies is based on the protective efficacy of rabies virus monoclonal neutralizing antibodies. Also, vaccinia recombinant viruses or DNA constructs that express only the envelope glycoprotein provide excellent protection, which is proportional to the titer of neutralizing antibody.

It is likely that antibody acts at several different levels, at the site of virus injection, at the neuromuscular junction and even within the central nervous system (CNS). Thus, if antiserum is applied at the site of rabiesvirus injection, it will reduce mortality. Specific depletion of antibody responses, by treatment with anti-μ antiserum, will potentiate intracerebral infection with an attenuated non-lethal rabies virus, implying that antibody can even reduce trans-synaptic transmission within the CNS.

Post-exposure immunization

Passive antibody, given shortly after infection with street rabies virus, does not reduce overall mortality, but does

Immunization status	Neutralizing antibody titer at challenge	Mortality dead/total	Mortality percent
Unimmunized	<2	14/17	82
Immunized	<2	8/10	80
	3–9	2/5	40
	10–99	4/18	22
	100–999	0/21	0
	>1000	1/13	8

TABLE 17.7 Protection against rabies conferred by pre-exposure immunization correlates with neutralizing antibody levels at the time of challenge. Monkeys were immunized with rabies vaccine and were then challenged intramuscularly with 10^5 mouse ic LD50 of street rabies virus
After Sikes RK, Cleary WF, Koprowski H, Wiktor TJ, Kaplan MM. Effective protection of monkeys against death from street virus by post-exposure administration of tissue-culture rabies vaccine. *Bulletin of the World Health Organization* 1971, 45: 10, with permission.

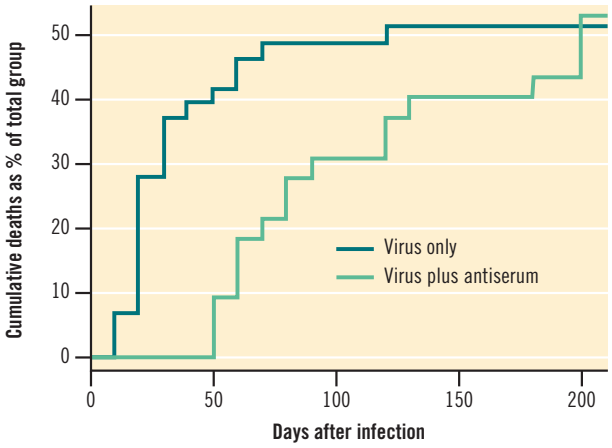

FIGURE 17.5 Anti-rabies virus antiserum prolongs incubation period without reducing mortality. Two groups of mice were infected by footpad injection of a street rabies virus with a long incubation period (17–120 days) and one group was given an intramuscular dose of a high titer equine anti-rabies virus antiserum (calculated to provide a neutralizing titer in the recipient of ~1:1000) one day after infection. The cumulative mortality in each group is plotted as a per cent of all the animals in the group. At 50 days, the treated group had experienced little mortality, compared to ~35% mortality in the virus only group, but eventually the cumulative mortality rose to ~50% in both groups. After Baer GM, Cleary WF. A model in mice for the pathogenesis and treatment of rabies. *Journal of Infectious Diseases* 1972, 125: 520–532, with permission.

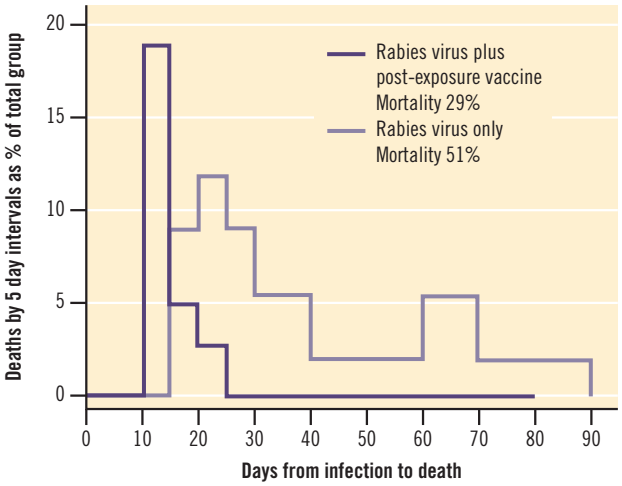

FIGURE 17.6 Post-exposure immunization against rabies can produce early death as well as partial protection. Mice were infected with 300 ic LD50 of street rabies virus. One group (43 animals) was untreated (mortality 51%), while the other group was immunized with inactivated rabies virus vaccine one day after infection (mortality 29%). Although the vaccine clearly reduced mortality, it also accelerated the time to death in animals that were not protected. After Baer GM, Cleary WF. A model in mice for the pathogenesis and treatment of rabies. *Journal of Infectious Diseases* 1972, 125: 520–532, with permission.

prolong the incubation period (Figure 17.5). Active immunization, begun just after infection with street rabies virus, will reduce overall mortality and passive antibody will synergize this protective effect, reducing mortality even further. This synergistic effect is likely due to the ability of antibody to delay virus spread thereby providing the host an advantage in the 'race' between the virus and induction of an active immune response.

Since active immunization elicits both antibody and cellular immune responses, which is responsible for the protective effect? Likely, both arms of the immune response play a role. If mice are immunized by intracerebral vaccine administration and challenged by the same route, there is a correlation between the degree of protection and cytolytic T lymphocyte (CTL) activity. Although this is a somewhat contrived experimental system conducted with primitive CTL assays, it does suggest that, in addition to antibody, CD8+ T cells can play a role in the outcome of rabies virus infection of the CNS.

Finally, rabies immunization illustrates a much-discussed but probably rare phenomenon, immune-mediated disease enhancement by use of a vaccine (Figure 17.6). In this example, the number of long incubation period cases was markedly reduced, but there was an absolute increase in short incubation period cases. The excess of short incubation period cases implies immune enhancement, although the mechanism awaits elucidation.

Hepatitis B virus (HBV)

The pathogenesis of HBV is characterized by a number of unusual features. The virus replicates mainly, perhaps exclusively, in the liver, where it produces a large amount of viral envelope (HBs) protein (>10^{10} filamentous and spherical particles per ml plasma) together with a smaller number (10^6 per ml plasma) of infectious virions. The course of acute hepatitis B is shown in Figure 17.7, with replication in the liver, rising levels of circulating HBs, induction of an immune response that leads to waning of HBs and the appearance of anti-HBs antibodies. The resolution of infection is accompanied by acute hepatitis that ranges from subclinical to severe or even fatal. The timing of events suggests that HBV is not cytopathic and that the acute hepatitis is caused by the cellular immune

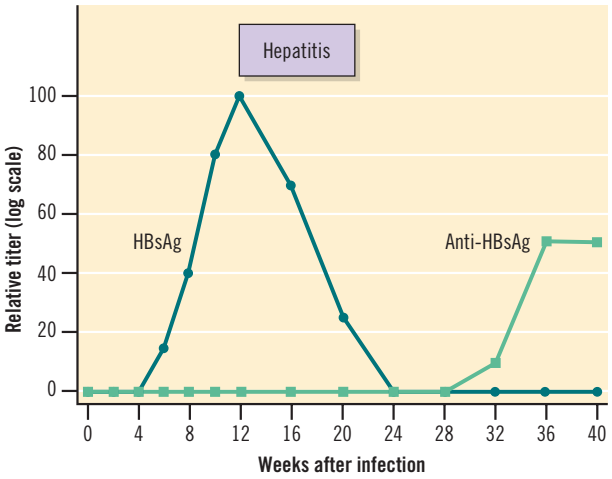

FIGURE 17.7 Course of HBV infection. In this example, HBV produces an acute infection with clinical hepatitis, following which the virus is cleared and the illness is resolved leaving antibodies against HbsAg as a permanent footprint of infection.

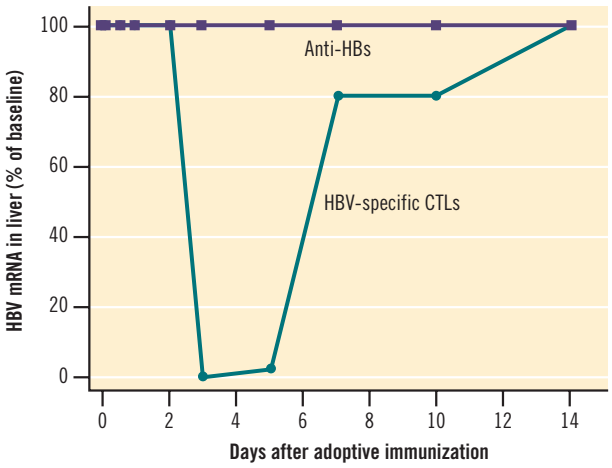

FIGURE 17.8 Clearance of HBV infection is mediated by CD8 T lymphocytes and not by anti-HBs antibodies. In this transgenic model, HBs antigen is expressed in hepatocytes and is shed into the blood. Congenic non-transgenic mice are immunized with HBs and used as a source of T cell clones specific for HBs as well as for anti-HBs antisera. T cells clear the liver, although only transiently, presumably due to the exhaustion of the transferred immunocytes. Other experiments indicate that clearance is mediated by cytokines (IFNγ and TFNα) secreted by the CD8 cells and not by CTL-mediated cytolysis, explaining how the mice survive the clearance process (see Figure 6.8). After Guidotti LG, Ando K, Hobbs MV *et al.* Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in transgenic mice. *Proceedings of the National Academy of Sciences* 1994, 91: 3764–3768, with permission.

response (see Chapter 7). An alternative course of infection, the persistent carrier state, is seen frequently in infants infected during birth. Such persistent infections are not accompanied by acute hepatitis, strengthening the view that infection alone does not cause hepatitis. However, neonatal infection carries a high risk of cirrhosis and hepatocellular carcinoma that develop decades later.

Evidence for the immune mediation of viral clearance is provided by the use of a transgenic mouse model, in which mice express one or several HBV proteins in the liver. When these animals are adoptively immunized with HBs-specific T lymphocytes, the viral protein is cleared from hepatocytes (Figure 17.8), but treatment with anti-HBs antibody has no effect. CD8-initiated viral clearance is mediated by cytokines (IFNγ and TNFα) secreted by effector cells that inhibit HBs expression, rather than by cytolysis, explaining how it occurs in the absence of overwhelming hepatitis (see Figure 6.8). By inference, it is likely that persistent infection represents a state of HBs immune tolerance due to 'exhaustion' or 'deletion' of HBs-reactive CD4+ and/or CD8+ T cells.

There is an effective HBV vaccine that consists of a recombinant form of the HBs antigen expressed in a eukaryotic cell system. It is often asserted that this vaccine protects by inducing anti-HBs antibodies that neutralize the virus. This is based in part on the protective effect of hepatitis B immune globulin (HBIG). In certain developing countries, a high proportion of women of childbearing age are chronic carriers of HBV and frequently transmit infection to their infants during delivery, with the subsequent development of persistent infections at a high frequency. If infants are given repeated doses of HBIG beginning at birth, several effects are observed: a small proportion (~10%) are totally protected from infection and a large proportion (~60%) undergo a short-term self-limited immunizing infection (Table 17.8). When this latter group is followed sequentially, circulating levels of passive antibody are seen for the first 6 months; when antibodies wane, there is a transient appearance of HBs antigenemia at 6–9 months, followed by the development of an active anti-HBs antibody response.

Outcome	Per cent distribution of subjects according to outcome		
	HBIG Treated (57)	Placebo (61)	Protection
HBs persistent *No anti-HBs*	26	95	None
HBs transient *Anti-HBs persistent*	58	0	Partial
No HBs *No anti-HBs*	11	0	Complete (uninfected)
No HBs *No anti-HBs*	5	5	Not exposed (uninfected)
Total	100	100	

TABLE 17.8 Protection of infants against perinatal HBV carriage by passive antibody (HBIG hepatitis B immune globulin) treatment at 0, 3 and 6 months. HBIG conferred varying degrees of protection, ranging from complete ('sterilizing' immunity) to none, with the majority of subjects being partially protected. It is assumed that the 16% uninfected subjects in the HBIG treated group include some babies who were completely protected and some who were unexposed
After Beasley RP, Hwang LY, Stevens CE *et al.* Efficacy of hepatitis B immune globulin for prevention of perinatal transmission of the hepatitis B virus carrier state: final report of a randomized double-blind placebo-controlled trial. *Hepatology* 1983, 3: 135–141, with permission.

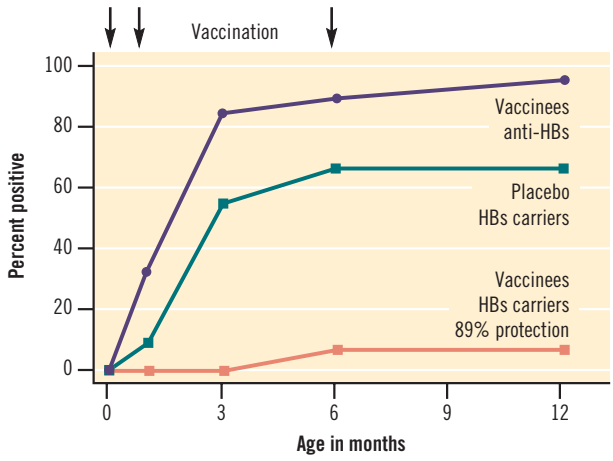

FIGURE 17.9 Prevention of the HBV carrier state in vaccinated infants. Babies born to mothers who were HBs carriers were divided into placebo and vaccinated groups and the vaccinees were given a whole virus inactivated vaccine at 0, 1 and 6 months. Immunization induced anti-HBs antibodies that appeared at 1–3 months postpartum. In spite of the delayed appearance of the antibodies, immunization markedly reduced the proportion of infants who became carriers. After Xu ZY, Liu CB, Francis DP *et al.* Prevention of perinatal acquisition of hepatitis B carriage using vaccine: preliminary report of a randomized double-blind placebo-controlled and comparative trial. *Pediatrics* 1985, 76: 713–718, with permission.

Since HBV infection is probably confined to the liver, it appears unlikely that passive antibody alone could clear the established liver infection that is signaled by antigenemia at 6–9 months. More likely, passive antibody reduces the initial infecting inoculum in the early postpartum period and this tips the balance so that the newly infected host is now able to mount an active response that clears the infection. From studies on transgenic mice, quoted above, it appears likely that clearance requires participation of the host's effector T lymphocytes. This scheme of immunization resembles an outmoded form of protection called 'passive-active' immunization, which employed passive antibody to reduce the dangers of active immunization with a pathogenic virus.

With this reconstruction in hand, we turn to data on the active immunization of infants against HBV. When infants born of mothers who are HBV carriers are immunized with an inactivated whole virus (or recombinant HBs) vaccine at birth and thereafter, a remarkable result is seen (Figure 17.9). Once again, a high proportion (~90%) of infections are 'converted' from persistent to acute and self-limited. This is surprising because the immune response to the vaccine only appears 1–3 months after birth (i.e. 1–3 months after infection). The sequence of events strongly implies that HBV infection is established in the liver and is subsequently cleared. Again, it seems likely that a host cellular immune response, elicited by either the vaccine or by the active infection, plays a role in vaccine-induced protection. In summary, a speculative reconstruction invokes the synergistic cooperation of both humoral and cellular immunity to explain the efficacy of HBV vaccine.

New vaccines

Several new human viral vaccines have undergone successful efficacy trials and illustrate some mechanisms of vaccine-induced protection.

Rotavirus vaccine

Rotaviruses are an important cause of infant diarrhea and death, particularly in developing countries. Rotaviruses have double-stranded 11-segmented RNA genomes and genetic reassortants are readily obtained from mixed infections. The pathogenesis of rotavirus disease is not completely understood, but at least two mechanisms have been identified. The virus infects and kills epithelial cells at the tips of intestinal villi and an internal protein, NSP4, acts as an enterotoxin. In addition, rotaviruses can also produce a transient viremia, but it is unclear what role this plays in disease causation. Rotaviruses have triple-layered virions, with two outer proteins, VP4 and VP7, both of which are targets for neutralizing antibody. These proteins also determine serotype; the most common VP7 serotypes are G1–G4 and G9 (G, glycoprotein) and the most common VP4 serotypes are P1 and P2 (P, protease sensitive). Vaccine trials (see below) suggest that there is some degree of immunological cross-protection between the different serotypes. Neutralizing antibody appears to be the most important determinant of protection against re-infection, while both T and B cells are important in recovery from primary infection.

Three live rotavirus vaccines have been developed, Rotashield (Wyeth), RotaTeq (Merck) and Rotarix (GlaxoSmithKline). Rotashield and RotaTeq are reassortant viruses, based on animal rotaviruses with VP4 and VP7 genes derived from human rotaviruses. Rotashield has a simian rotavirus and RotaTeq a bovine rotavirus backbone. By contrast, Rotarix is a single human rotavirus (serotype G1 P1) that was attenuated by passage in cell culture. The ability of these three vaccine viruses to replicate in the human enteric tract varies considerably, in a high-to-low hierarchy, from Rotarix to Rotashield to Rotateq. The dose used for immunizing human infants is highest for Rotateq and lowest for Rotarix. These vaccines are administered in two or three oral doses, beginning at age 2 months. The vaccines elicit intestinal IgA and vaccine 'takes' are usually determined by detection of virus-specific serum IgA. In large scale trials, all three vaccines have been >80% efficacious at preventing severe rotavirus diarrheal disease in young infants.

Rotashield was the first of these vaccines to be licensed but was withdrawn because it caused intussusception (a telescoping of the small intestine causing gangrene and peritonitis, requiring surgical intervention). The excess of intussusception cases (about 1 case per 10 000 vaccinees) occurred mainly during the first 2 weeks after the first dose of vaccine. Although the etiology of intussusception is not known, it has been speculated that the vaccine virus causes transient inflammation and swelling of Peyer's patches (lymphoid follicles in the intestinal wall) and that peristalsis leads to mechanical internalization of an intestinal segment. The other two rotavirus

vaccines have not been associated with intussusception. RotaTeq was licensed in the USA in 2006 and it is expected that Rotarix will be licensed in the near future.

Mucosal immunity

Rotavirus vaccines raise provocative questions associated with mucosal immunity.

In contrast to most ingested foreign proteins, why are the viral proteins immunogenic? This paradox is not completely understood, but it appears that there are several factors that favor immune induction in response to selected ingested antigens. Rotaviral infection of the intestinal tract is an invasive process that elicits an innate and acquired immune response, in contrast to the passive presence of a foreign protein in the intestinal lumen. Invading viral RNA will bind to TLRs 3, 7, 8 (see Table 5.2), activating dendritic cells and facilitating immune induction. Rotavirions are taken up by activated dendritic cells in the intestinal epithelium and these professional APCs process viral proteins for presentation to T cells.

Is vaccine-induced protection due to local virus-specific IgA? Antiviral IgA can be identified in the intestinal secretions of immunized infants and likely neutralizes ingested rotaviruses. However, rotaviruses also produce a transient viremia and protection against severe disease may be partly due to circulating antiviral IgG, which the vaccines also induce. There is no clear evidence whether cellular immune responses play a role in vaccine-induced protection against rotaviral disease.

Human papillomavirus (HPV) vaccine

HPV has evolved to replicate in a very specialized niche, i.e. the epithelium of skin and mucous membranes (see Chapter 4). There are over 100 HPV serotypes and a few of them (particularly types 16, 18) are a significant cause of cervical cancer (see Chapter 12). It is estimated that, combining all serotypes worldwide, HPV causes at least 200 000 cervical cancer deaths annually. Following infection with oncogenic types of HPV, cervical cancer develops in a series of steps progressing from initial infection, to persistent infection, to hyperplasia, to cervical intraepithelial neoplasia (CIN), to cervical cancer in situ, followed by metastatic spread. The whole process takes many years but the early phases can be detected within 1–2 years of infection in some subjects.

HPV vaccines have been formulated to prevent or ameliorate infection with HPV and are not directed against the oncogenic proteins (E6 and E7) of the virus. HPV has an outer capsid and the L1 protein is its major component. When L1 is expressed as a recombinant protein, the monomers self-assemble into virus-like particles and these particles will induce serum neutralizing IgG when administered as a parental immunogen. Neutralizing antibodies and protection are mainly type specific, so that vaccines are formulated as multivalent products.

IgG antibodies also appear in cervical secretions at titers that range from 10% to 1% of those in serum, depending on the phase of the ovulatory cycle. Vaccine-induced titers of serum neutralizing antibodies are mainly in the range 100–10 000 so that titers in cervical secretions would be predicted to range from a minimum of 1–100 to a maximum of 10–1000. If sexual contact produces local mucosal trauma, the concentration of serum antibodies could be higher at the contact site.

There are two candidate L1 vaccines (Merck and GlaxoSmithKline) that have been tested in phase 2 trials. The vaccines induce circulating neutralizing antibodies in a high proportion of vaccinees and they also have shown a high degree of efficacy against the earliest oncogenic changes (Table 17.9). It appears likely that both vaccines will be licensed in the USA in 2006 or 2007.

Mechanisms of protection

Natural history studies of oncogenic types of HPV shows that infections are readily transmitted among young adults once they become sexually active. For instance, one study of college students showed that about 60% of seronegative women became seropositive during a 5-year follow up. When infection is measured by PCR-based detection of viral DNA in cervical secretions, it appears that persistence of HPV is variable and many infections are spontaneously cleared, presumably by the host immune response. Furthermore, it is likely that progression from infection to cervical carcinoma requires HPV persistence for years, so that the lifetime risk of cancer among seropositive women is $<1\%$. This implies that it might be

Treatment group	Number of subjects	Women-years at risk	Event	Number of events	Incidence per 100 women-years at risk	Vaccine efficacy
Placebo	233	533	Persistent infection	36	6.7	
Vaccine	235	566	Persistent infection	4	0.7	90%
Placebo	233	533	Lesions	6	1.1	
Vaccine	235	566	Lesions	0	0	100%

TABLE 17.9 Protection against early steps in cervical carcinoma conferred by an HPV vaccine. Young women were immunized with quadravalent HPV vaccine (types 16, 18, 6, 11) at 0, 1 and 6 months and followed for 30 months thereafter. Subjects were assessed for persistent infection (by repeated detection of HPV DNA in cervical swabs and tissue biopsies) and for virus-positive lesions (cervical intraepithelial neoplasia or external genital warts)
After Villa L, Costa RLR, Petta CA *et al.* Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomized double-blind placebo-controlled multicentre phase II efficacy trial. *Lancet Oncology* 2005, 6: 271–278, with permission.

possible to reduce the risk of cancer with a vaccine that induced sterilizing immunity or reduced viral persistence.

The L1-based HPV vaccines have shown an unexpectedly high degree of efficacy and the mechanisms of protection are only partially understood. It appears that the vaccine may protect at several levels:

1. circulating anti-HPV IgG antibodies appear as a transudate in the female genital tract
2. genital tract antibodies reduce the frequency of infection in young women exposed to HPV
3. if HPV infection is not prevented, antibodies may reduce lateral spread of infection in the epithelium
4. HPV infections vary in the length of persistence and antibodies probably reduce the proportion of infections that persist (persistence is a necessary requirement for the stepwise oncogenic pathway)
5. passive antibody studies indicate that IgG induced by virus-like particles could account for protection.

These considerations provide at least a partial explanation how a vaccine that induces serum IgG could provide effective protection against a mucosal infection acquired by sexual contact.

AIDS VACCINE

History and challenges

The effort to develop an AIDS vaccine represents a landmark in the history of viral vaccines. AIDS is arguably the greatest public health challenge of the first part of the 21st century and it is broadly held that any effective strategy to control this global pandemic will depend upon an effective vaccine. However, development of a safe and effective vaccine presents a much more difficult scientific challenge than any prior viral vaccine.

When HIV was identified as the cause of AIDS in 1983/84, it was immediately recognized that vaccine development was an important priority. Yet, more than 20 years later, we lack a vaccine in spite of the greatest investment that has ever been made in vaccine research. How did we blunder into this impasse? There are several reasons for the present dilemma. Initial efforts were premised on the assumption that it would be possible to formulate an AIDS vaccine based on past successes. Almost all successful viral vaccines have utilized one of two approaches, either a live attenuated variant virus or an inactivated virus or viral protein.

HBV vaccine, introduced in 1986, was formulated as a recombinant form of the major glycoprotein of HBV. Coming to fruition concomitant with the isolation of HIV, the outstandingly successful HBV vaccine suggested that a similar approach could be applied to HIV, a virus that also has a single viral attachment glycoprotein. Implicit in this approach was the assumption that immunization with the protein would induce neutralizing antibodies. Unfortunately, HIV did not follow the pattern of HBV, for several reasons. First, both natural infection with HIV or immunization with recombinant gp120 induces antibodies, but these have little or no ability to neutralize the virus. Second, when formulated as an immunogen, the recombinant HIV envelope protein is a monomer that does not readily form trimers, which are the native macromolecular form found on the mature virion. The monomer lacks some of the conformational neutralizing epitopes found on the trimer and induces antibodies that will bind the monomer but not neutralize infectious HIV. Finally, a recent landmark phase III efficacy trial of monomeric gp120 showed that it lacked protective efficacy.

The other approach, development of an attenuated variant virus, has also been explored in some depth but the results have been disappointing. A naturally attenuated strain of HIV was discovered in Sydney, Australia, to have infected a cohort of about 10 persons who had received contaminated blood transfusions. This variant virus had a major deletion in the *nef* gene, which is known to attenuate SIV (simian immunodeficiency virus). For about 10 years the 'Sydney cohort' followed a benign course similar to that seen in non-progressors infected with HIV, producing optimism about the outlook for a safe attenuated HIV variant. However, between years 10–15 after infection, most members of the Sydney cohort began to lose their CD4 cells and showed other evidence of incipient albeit long incubation period AIDS. This experience has had a chilling effect on any hope to develop a safe attenuated HIV strain for use as a human vaccine.

Thus, more than 20 years after isolation of HIV, it has become clear that an empirical approach to the development of an AIDS vaccine has failed. Furthermore, there is a paucity of information about the mechanisms by which established effective viral vaccines confer their protection. The remainder of this section focuses on the immunobiological issues critical for the development of an AIDS vaccine.

The macaque model

Simian immunodeficiency virus (SIV) denotes a group of viruses isolated from various African monkey species. The SIVs appear to be non-pathogenic in their natural host species, but some isolates cause AIDS in Asiatic macaques. Furthermore, it is possible to construct recombinants between SIV and HIV that have the HIV *env* gene inserted into an SIV genetic backbone (simian-human immunodeficiency virus or SHIV) and some of these viruses also cause AIDS in macaques. There are a number of variables that influence the degree of pathogenicity of SIV or SHIV, including viral variation, dose and route of infection and animal-to-animal variation in susceptibility. Thus, it is possible to simulate the spectrum of infections that are seen in humans. Data from the macaque model have become critical for the development of an AIDS vaccine and for analysis of mechanisms of protection.

Will partial immunity protect against AIDS?

Natural infection with wild type virus provides excellent long-lasting protection against re-challenge with the

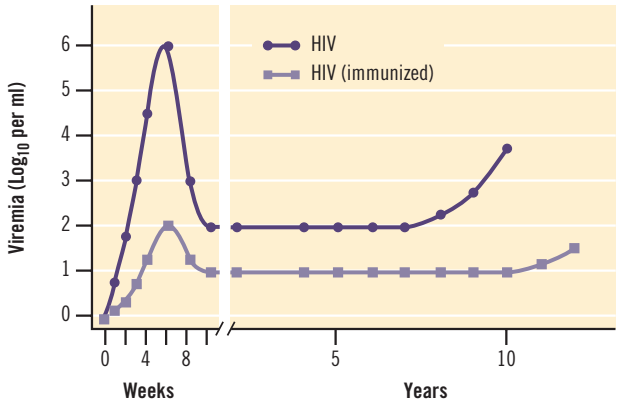

FIGURE 17.10 The dynamics of HIV infection, illustrating the challenges to a prophylactic vaccine. Most viral vaccines are directed against acute infections and an immunized subject is successfully protected if the extent of replication is reduced for a period of days or a few weeks. However, reduction of acute HIV infection will not prevent the establishment of a persistent infection. In contrast to many other persistent infections that can be innocuous, HIV may eventually erode the CD4 lymphocyte population sufficiently to produce immunodeficiency, albeit with an extended incubation period.

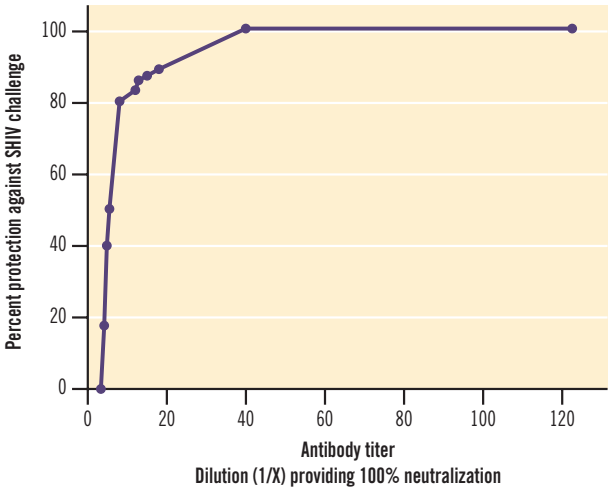

FIGURE 17.11 Circulating neutralizing antibody present at the time of exposure can down modulate SHIV infection of macaques. Monkeys were given intravenous injections of immunoglobulin that did or did not contain antibodies against HIV and one day later were challenged intravenously with a pathogenic dual-tropic SHIV DH12. The neutralizing titer in the plasma of passively immunized monkeys was determined just before challenge. A passive antibody level of ~1:40 protected 100% of animals and lower titers provided partial protection. Antibody titers are expressed as the highest dilution of plasma that neutralized 100% of 100 TCID (tissue culture infectious doses) of the same virus used for infection. After Nishimura Y, Igarashi T, Haigwood N *et al.* Determination of a statistically valid neutralization titer in plasma that confers protection against Simian-Human immunodeficiency virus challenge following passive transfer of high-titered neutralizing antibodies. *Journal of Virology* 2002, 76: 2123–2130, with permission.

same virus and constitutes the 'gold standard' for immune-mediated prophylaxis. However, even natural infection does not provide 'sterilizing' immunity, which is rarely seen with effective vaccines (for an exception see Table 17.9). It may be questioned whether an AIDS vaccine that produces only partial protection will prevent the occurrence of AIDS following exposure to wild type HIV. Figure 17.10 illustrates the problem. To prevent disease, vaccines that protect against acute virus diseases need only modulate the degree of spread or replication for a limited period of day to weeks. An AIDS vaccine of similar efficacy might reduce the viral setpoint but would not prevent the persistence of infection. Would this down modulated but persistent infection eventually cause AIDS?

When the most immunogenic experimental SIV vaccines are tested in the macaque model, the immunized animals often show a reduction of ~100-fold in their viremia titers relative to control monkeys, but SIV persists. This is analogous to differences seen in human cohort studies such as illustrated in Figure 14.8, in which HIV-infected subjects were divided into four quartiles according to their viral setpoint at 6–9 months after infection and their AIDS-free survival plotted. In the quartile with the highest titer, 90% had developed AIDS in 10 years, while only 10% of the quartile with the lowest setpoints had developed AIDS in 10 years. HIV-2 is also illustrated in Figure 14.8 to show that with an even lower setpoint, a considerable proportion of infected persons do not develop AIDS within their lifetimes. At present, we do not know which paradigm might apply to an AIDS vaccine.

Correlates of protection

In the development of a vaccine, it is very useful to have an immunological correlate of protection, which can be used to evaluate different vaccine formulations and immunization schedules. A vigorous search for such a correlate has been made in the macaque model and a large literature is replete with conflicting claims. The very inconsistency in claims suggests that there is no single correlate of protection. Instead, several different immune modalities may synergize to provide protection (the 'barrier' hypothesis).

Evidence that both cellular immunity and antibody could contribute to an effective AIDS vaccine comes from the macaque model. If monkeys persistently infected with SIV are depleted of their CD8 cells, their viremia levels rise rapidly only to drop when CD8 cells are reconstituted (see Figure 14.11). Neutralizing antibodies also can down modulate an experimental infection as shown by the effect of pre-treatment of macaques with immune globulin prior to challenge with SHIV (Figure 17.11). Thus there is persuasive evidence that several immune modalities could contribute to a protective AIDS vaccine.

Recent studies of superinfection have contributed important data regarding protection against HIV infection. Superinfection is defined as the de novo appearance of a second genetically distinct strain of HIV-1 in an infected patient who has been tested periodically. Salient observations in several studies found that superinfections occurred at a frequency of about 2–5 per 100 person-years in patients during the first 1–2 years after first HIV infection and were not detected in patients after that time, in spite of documented exposure to partners

with genetically distinct strains of HIV-1. Among recently infected patients, those who experienced superinfections had little if any neutralizing antibody (against autologous and heterologous strains of HIV-1), while patients who did not experience superinfection had detectable if modest antibody titers. These data suggest that an AIDS vaccine that induced broad neutralizing antibody might provide significant protection against HIV infection.

Induction of neutralizing antibody and cellular immunity

HIV differs from most viruses in that infected persons, with some exceptions, develop only minimal levels of neutralizing antibody. Sera from infected patients contain antibodies against the viral attachment (env) proteins but there is little neutralizing activity. Although many anti-gp120 monoclonal antibodies have been isolated from HIV-infected persons, only a few of them have broad neutralizing activity. During virion budding, SIV incorporates MHC class II proteins into the virion envelope (in addition to the viral envelope proteins) and antibody against the MHC molecule has potent neutralizing activity. Taken together, these observations suggest that the difficulty in inducing neutralizing antibodies is due to the properties of the gp120 molecule, the viral attachment protein.

Resistance to neutralization is explained in part by the structure of gp120, which has a large number of glycosylation sites (~24 typically), so that the virion spike is partly shielded from antibodies. In theory, the CD4 binding site on pg120 should be a conserved target for neutralizing antibodies. However, the configuration of the CD4-binding site makes it inaccessible to the antigen-binding domain on most antibody molecules. In addition, there are several loops (particularly the variable regions 1 and 2) that partly cover the CD4-binding site. These structural features are presumed to impede both the induction and the action of neutralizing antibodies.

The notorious mutability of HIV also contributes to its ability to escape neutralization. When autologous neutralization tests are conducted on HIV-infected patients who have been followed from the time of infection, neutralizing antibodies are induced. However, there is a continual selection of viral escape mutants that continues for years until broadly neutralizing polyclonal antibodies eventually develop (Table 17.10). This pattern has also been seen with other lentiviruses, such as equine infectious anemia and maedi-visna viruses, in their natural hosts.

By contrast, it appears relatively easy to induce cellular immunity to HIV. Infected patients have CD8 cells that are responsive in various assays for cellular immunity such as the Elispot and intracellular cytokine assays (see Chapter 14). Epitopes can be mapped to most of the viral structural genes (about 15 epitopes in total per patient), with the highest proportion of responses to the gag proteins. Also, a variety of candidate immunogens are capable of inducing CD8 responses in macaques, with partial protection against AIDS. However, SIV and HIV escape mutants can permit the virus to circumvent a potent cellular immune response.

	Neutralizing titer of plasma obtained at indicated months after infection			
Virus isolate months after infection	0 months	6 months	12 months	18 months
0	*<100*	675	2670	2190
6	<100	*<100*	1769	2247
12	<100	<100	*<100*	556
18	<100	<100	117	*122*

TABLE 17.10 HIV-1 constantly evolves to escape neutralization by the patient's antibodies. In this example, virus isolates from a single patient were tested in a checkerboard against sera from the same patient. The patient did produce neutralizing antibodies but their activity was limited to virus isolates obtained at prior time points

After Richman DD, Wrin T, Little SJ, Petropoulos CJ. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proceedings of the National Academy of Sciences* 2003, 100: 4144–4149, with permission.

Cross-clade immunity

HIV-1 viruses can be classified into about 10 genotypes, usually called clades, all of which are present in Africa but which have quite different distributions in the Western hemisphere, Europe and Asia. Within a given clade, there is less than 20% nucleotide diversity. There is up to 35% diversity between clades, which is greatest in the *env* gene and less in the *gag* and *pol* genes. Originally, it was postulated that clades might represent immunotypes and that it would be necessary to formulate a multivalent vaccine for use in different geographic areas. However, it now appears that clades do not represent immunotypes and that there is a considerable degree of cross-clade immune responses. If HIV-infected subjects are tested and those few sera are selected that exhibit some degree of homologous neutralization, most also show cross-clade activity (Table 17.11). Likewise, CTLs from infected subjects show both homologous and heterologous activity.

Candidate AIDS vaccines

There are several candidate vector-based AIDS vaccines that are now in early human trials. These candidate vaccines have been tested in macaques, followed by challenge with virulent SHIV or SIV. Figure 17.12 shows one example that uses a DNA vaccine prime followed by a recombinant vaccinia virus boost and Figure 17.13 shows another that uses a DNA prime followed by a boost with a recombinant adenovirus vector. Neither of these vaccine regimens prevents infection, but both of them attenuate infection with the virulent challenge virus.

	Log 2 neutralization titer versus virus of clade									
Sera	A	B	C	F1	E	F2	G	H	O2	O3
A	5	0	0	0	0	0	0	0	0	0
B	11	8	9	7	11	11	8	7	11	11
C	7	6	7	7	8	9	7	3	8	8
E	7	5	7	6	7	7	6	4	6	7
F1	7	6	8	5	7	8	5	5	6	6
F2	7	6	7	3	8	8	4	3	8	7
G	6	5	7	6	7	8	6	4	5	4
H	8	6	7	4	8	8	6	5	5	8
O2	6	6	7	0	7	5	0	0	7	8
O3	0	0	0	0	0	0	0	0	0	6

TABLE 17.11 Cross-clade neutralization titers from a panel of 10 pairs of sera and isolates from patients infected with HIV-1. A panel of sera that showed significant neutralization of a clade-matched wildtype isolate was tested against isolates representing most other clades. Most sera showed cross-clade neutralization although there were some exceptions
After Nyambi PN, Nkengasong J, Lewi P *et al.* Multivariate analysis of human immunodeficiency virus type 1 neutralization data. *Journal of Virology* 1996, 70: 6235–6243, with permission.

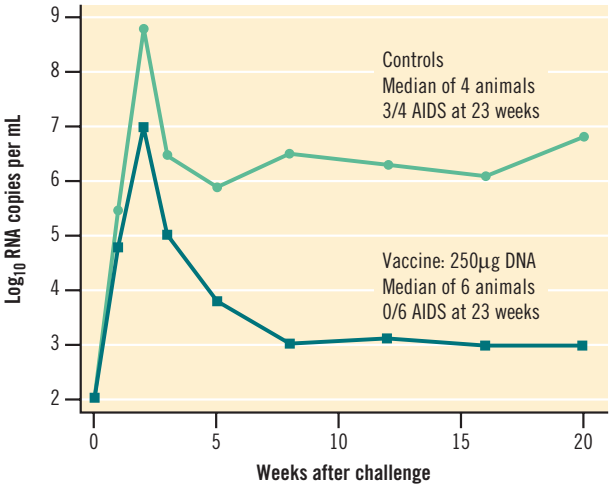

FIGURE 17.12 Protection against SHIV in macaques by a prime-boost mixed modality vaccine. These animal were immunized intradermally at 0 and 2 months with 250 μg of a DNA construct expressing most of the proteins of SHIV 89.6 and were boosted at 6 months with a recombinant MVA (attenuated vaccinia) virus expressing the three major proteins (gag, pol, env) of SHIV 89.6. At 13 months, they were challenged by intrarectal infection with pathogenic SHIV 89.6P. The vaccinated monkeys contained their viremia (median titer is shown) while the control infected animals did not. Not shown is the loss of CD4 lymphocytes in all of the control infected animals while 6/6 vaccinated animals maintained their CD4 counts. Since there is no cross neutralization between SHIV 89.6 and SHIV 89.6P, the considerable protection observed must be mainly attributed to cellular immunity. After Amara RR, Villinger F, Altman JD *et al.* Control of a mucosal challenge and prevention of AIDS in rhesus monkeys by a multiprotein DNA/MVA vaccine. *Science* 2001, 292: 69–74, with permission.

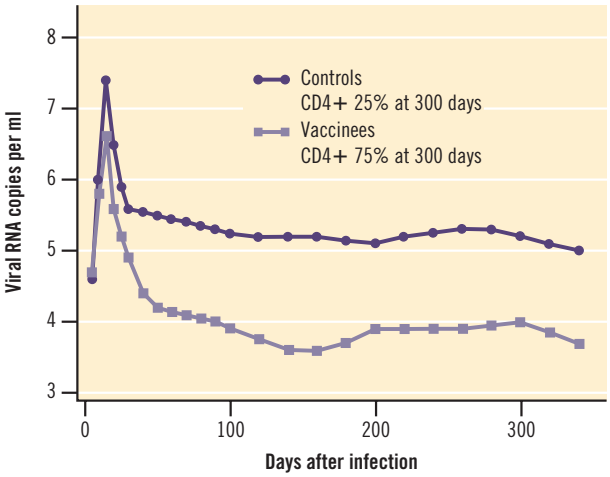

FIGURE 17.13 Partial protection of rhesus macaques against virulent SIVmac239 with an experimental immunogen containing *gag*, *rev*, *tat*, *nef* genes. The viral genes were formulated as a recombinant DNA (administered at weeks 0, 4, 8) and recombinant adenovirus (boost at week 24), followed by repeated low dose rectal challenge at 50 weeks. The immunogen induces only cellular immunity since it does not include the *env* gene. Vaccinated animals exhibit both lower plasma viral setpoint and relatively well-preserved CD4+ T cell levels in the blood. After Watkins D. Immune responses that successfully control AIDS virus replication. *Conference on retroviruses and opportunistic infections*, 2006, presentation number 180, with permission.

REPRISE

The mechanisms whereby immunization protects against virus disease depend upon the pathogenesis of the specific infection. In some instances, pre-formed neutralizing antibody intercepts invading virus at the portal of entry and partially or (rarely) totally inactivates the viral inoculum. In other instances, circulating antibodies neutralize virus entering the blood, preventing dissemination to key target organs or tissues. Antibody may even block transit of viruses by the neural route. In some instances, CD4, CD8, B lymphocytes and perhaps other lymphoreticular elements cooperate to provide vaccine-induced protection that is more effective than that mediated by any single component of the immune response. In vaccine-protected individuals, exposure to wildtype virus initiates a mild infection that is rapidly cleared and this clearance likely involves CD8 effector lymphocytes as well as antibody.

There are many methods to produce a viral vaccine. Most approved human vaccines are either live attenuated viruses or non-replicating immunogens (inactivated viruses or recombinant viral proteins). A number of new vectors are now under development as vaccine platforms. These fall into three classes:

1. attenuated viruses – such as vaccinia or other poxviruses that have been engineered to express additional viral proteins
2. viral replicons, which are non-replicating constructs that will introduce either RNA or DNA encoding viral proteins into host cells
3. 'naked' DNA plasmids that encode a protein immunogen under the control of specific promoter sequences.

Recent research on innate immunity has shown that immunological adjuvants act by stimulating toll-like receptors and these insights are being used to develop new adjuvant formulations that will enhance vaccine immunogenicity.

Different vaccine modalities may protect by inducing a different spectrum of immune defenses. Each vaccine modality has its advantages and disadvantages and it is unpredictable which approach will provide the most effective protection against a specific virus. The newer vector systems now under development may lead to safer vaccines, particularly if combined with the use of cytokines or other biological and chemical adjuvants.

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Glossary and Abbreviations

$\alpha_v\beta_6$ integrin chains
α-herpesviruses herpesviruses are classified as alpha, beta or gamma, based on their cellular tropism
A2G an inbred mouse strain
AAV adeno-associated virus, a parvovirus that depends upon adenovirus as a 'helper'
ACE2 angiotensin-converting enzyme 2
ADCC antibody-dependent cellular cytolysis
ADV Aleutian disease virus
AIDS acquired immunodeficiency syndrome
A/J an inbred mouse strain
Aleutian disease a disease caused by a parvovirus that is particularly pronounced in the Aleutian strain of mink
ALL acute lymphatic leukemia
ALT alanine transaminase, a liver enzyme whose level in the serum reflects liver function
ALV avian leukosis virus
ALV-A avian leukosis virus A
amphotropic a class of murine leukemia viruses that will infect cells from mice and other species
Anterograde in the context of neurons, away from the cell body and perinuclear region
Anti-HBs antibody against HBs antigen
AP-1 activating protein 1, a transcriptional activation complex of proteins
AP-2 adapter complex that recruits transmembrane proteins to clathrin-coated pits
APC adenomatosis polyposis coli
APCs antigen-presenting cells
APOBEC apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like, a family of cytidine deaminases
Apoptosis programmed cell death, in contrast to necrosis
Arboviruses arthropod-borne viruses that are transmitted by an insect vector to a vertebrate host
ASC antibody-secreting cell
ASLV avian sarcoma leukemia virus
ASV avian sarcoma virus
ATL acute T cell leukemia
Av01 an attenuated mutant rabiesvirus selected by a neutralizing monoclonal antibody

B19 designation of a human parvovirus that infects erythrocytes
B8R a poxvirus protein homologous to the IFNγR
B cell lymphocyte that has matured in bone marrow
BALB/c an inbred mouse strain
Bcl-2 anti-apoptotic molecule
Bgp1 biliary glycoprotein 1, a cellular membrane protein that also serves as a receptor for mouse hepatitis virus
BHK-21 a continuous line of baby hamster kidney cells
BK polyomavirus a human polyoma virus, named after a patient from whom it was isolated
BLV bovine leukemia virus
BrdU bromodeoxyuridine, a nucleotide used to label cells

C3, C1q, C4b complement proteins
C4b a complement protein, an intermediary in the complement cascade
C4b-BP plasma protein that binds C4b
CA capsid protein of retroviruses
Carbolic acid dilute phenol, an antiseptic
Carcinoma cancer
CAT cationic amino acid transporter
CAT1 cationic amino acid transporter
CC cysteine-cysteine chemokines
CC chemokines chemokines with a cysteine-cysteine motif
CCR5 a chemokine receptor that also serves as a co-receptor for HIV
CD4 cluster of differentiation 4, a cell surface marker defined by anticellular antibodies, used to define helper T lymphocytes
CD8 cluster of differentiation 8, a cell surface marker defined by anticellular antibodies, used to define effector T lymphocytes
CD11a adhesion molecule
CD25 component of IL-2 receptor
CD27 TNF receptor superfamily
CD43 adhesion molecule
CD44 adhesion molecule
CD46 receptor for measles virus
CD62L lymph node homing receptor
CD69 early activation marker
CD127 IL-7 receptor α chain
CDK cyclin-dependent kinase
CDV canine distemper virus
CEACAM carcinoembryonic antigen-related cell adhesion molecule
CEV cell-associated enveloped poxvirus
CIN cervical intraepithelial neoplasia
CMV cytomegalovirus, a γ-herpesvirus
c-myc cellular myoclastosis proto-oncogene, a transcription factor
CNS central nervous system
Colchicine a drug that can block fast axoplasmic transport in neuronal processes
Complement fixing antibody antibodies that are capable of binding complement through the Fc domain on the antibody molecule
Condyloma wart
Coronaviridae a family of positive-stranded RNA viruses
CoV coronavirus
CpG motifs unmethylated DNA sequences that are recognized by TLR9, inducing innate immune responses
CPV canine parvovirus

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CR1, 2 conserved region 1 and 2, in adenovirus E1A and HPV E7 proteins
CREB CRE binding motif; CRE cAMP responsive element; cAMP cyclic adenosine monophosphate
CRPV cottontail rabbit papilloma virus
CTAR C-terminal activator regions 1 and 2
CTL cytolytic T cells, lymphocytes capable of lysing target cells on an antigen-specific basis
Cupping therapeutic bleeding
CVDPV circulating vaccine-derived poliovirus
CVS challenge virus standard, a brain passaged highly neurovirulent strain of rabies virus
CXC chemokines chemokines with a cysteine-X-cysteine motif
CXCR4 4th receptor for CXC chemokines

Δ32 mutation a 32 base pair deletion in the CCR5 gene that results in lack of expression of the CCR5 protein on the cell surface
DC dendritic cell
Dendritic cells specialized macrophages found in skin and lymphoid tissues, that are 'professional' antigen presenting cells
DHF/DSS dengue hemorrhagic fever/dengue shock syndrome
DTH delayed-type hypersensitivity
Dysplasia pre-cancerous change in cell phenotype

E envelope protein
E, L early and late genetic regions
E1A, E1B, E3 two 'early' proteins of adenovirus
E2 one of the envelope proteins of Sindbis virus, an alphavirus
E2F family of related transcription factors
EBNA Epstein-Barr nuclear antigen
EBV Epstein-Barr virus
ecotropic a class of murine leukemia viruses that will only infect mouse cells
EGF epidermal growth factor
EIAV equine infectious anemia virus, an equine lentivirus that causes a characteristic acute anemia
EIF eukaryotic Initiation Factor
ELISA enzyme-linked immunosorbent assay
Elispot an assay for functional CD8 cells that measures the production and secretion of cytokines such as IFNα in response to a specific antigen
endogenous applied to retroviral sequences that exist as sequences within germline DNA
enhancer a DNA sequence that enhances transcription by binding cellular proteins that convert cellular DNA from a 'condensed' state associated with histones to an 'open' state that is accessible to RNA polymerases
env envelope gene of retroviruses
ER endoplasmic reticulum
ErbB-1 cellular growth factor
ESCRT-1, -2, -3 endosomal sorting complex required for transport
ev endogenous chicken virus, retroviral sequences in the chicken genome
exogenous applied to retroviruses that circulate as replication competent viruses and are transmitted as an infection from host to host

F1 progeny from a cross between two parental strains of an organism
FACS fluorescent activated cell sorter
FcγIIIR Fcγreceptor
FcR receptor for the Fc domain of immunoglobulin molecules
FeLV-B feline leukemia virus B
FeSV feline sarcoma virus
Flv gene that influences susceptibility to flaviviruses
FMD foot-and-mouth disease virus
FMR Friend, Maloney, Rauscher group of murine leukemia viruses
F-MuLV Friend murine leukemia virus
Fomites microbiologically-contaminated materials that transmit infection
FIV feline immunodeficiency virus, a lentivirus of cats
FPV feline panleukopenia virus

gag group antigen(s) of retroviruses
gag gene the gene that encodes the major internal structural proteins of retroviruses
GALT gut-associated lymphoid tissue
GALV gibbon ape leukemia virus
gB, gC, gD, gE, gI, gC glycoproteins of herpes simplex virus
GH growth hormone
G-MuLV Gross murine leukemia virus
gp41 HIV transmembrane (TM) envelope protein
gp120 HIV surface (SU) envelope protein
gp160 HIV envelope glycoproteins prior to cleavage into gp41 and gp120
GPCR G-protein-coupled receptor
Gross/AKR Gross subgroup of murine leukemia viruses
GSK glycogen synthase kinase
GTPase guanosine triphosphatase

H1N1 hemagglutin 1, neuraminidase 1, terminology indicating antigenic classification of influenza viruses
H5N2 designation for the hemagglutinin and neuraminidase of influenza virus, based on serotype classification of human and animal influenza viruses
HA hemagglutinin of influenza virus, consisting of two peptides, HA1 and HA2
HAART highly active antiretroviral therapy
HAV hepatitis A virus
HAVcr-1 HAV cellular receptor
HBcAg the core antigen of HBV
HBIG hepatitis B immune globulin
HBs hepatitis B surface antigen, the viral envelope protein
HBV hepatitis B virus
HCV hepatitis C virus
Heparan sulfate a glycosaminoglycan (complex carbohydrate)

HER hemorrhagic encephalopathy of rats
herd immunity protection conferred on unimmunized members of a partially vaccinated population because virus transmission is reduced in the immunized members of the group
HHV8 human herpesvirus 8, also called Kaposi's sarcoma herpesvirus
HIV-1, HIV-2 the two major types of human immunodeficiency virus
HLA human leukocyte antigen
HPCs hematopoietic precursor cells
HPV human papillomavirus, a cause of cervical carcinoma
HR heptad repeat
HSV herpes simplex virus
HTLV-I human T lymphocyte tropic virus type I
HVEM herpesvirus entry mediator, member of the TNF receptor family of proteins

ic intracerebral
ICAM intercellular adhesion molecule
ICE interleukin 1 converting enzyme
ICP'n' infected cell protein, a term used to designate individual proteins of HSV
IE immediate early, E (early) and L (late) genes of HSV; a listing of viral genes according to the time of their expression during replication
IEV intracellular enveloped poxvirus
IFN interferon
IFNαRKO interferon α/β knockout mice
IFNγ interferon gamma, or immune interferon
IFNγR cellular receptor for IFNγ
Ig immunoglobulin
IgA immunoglobulin A, an immunoglobulin that is secreted by B cells found in mucosal tissues
IgG immunoglobulin, once called gamma globulin
IL-1 interleukin 1
Il-2 interleukin 2 (also called T cell growth factor)
Il-4 interleukin 4
IL-Rγc interleukin 2 receptor γ chain
immortalized cell line cell line that can be maintained indefinitely in culture, in contrast to non-transformed cell lines that can be maintained for about 60 subcultures before they lose viability
IN integrase enzyme of retroviruses
INOS inducible nitric oxide synthase
In situ PCR a method for the histochemical identification of specific nucleic acids using sequence amplification followed by in situ hybridization
IntDCs interstitial DCs
IPV inactivated poliovirus
IRES internal ribosome entry site
ISVP infectious subvirion particle of reovirus

JAK the Janus tyrosine kinase
JC polyomavirus a human polyoma virus, named after a patient from whom it was isolated
JNK Jun N-terminal kinase

KIR killer inhibiting receptor, receptors in NK cells that inhibit perforin-mediated killing
'Knockout' mice mice in which a specific gene has been inactivated using a method that involves homologous DNA recombination in embryonic stem cells
KS Kaposi's sarcoma, a skin cancer
KSHV Kaposi's sarcoma herpes virus, the cause of KS, also called HHV8

L929 cells a murine cell line
LANA latency-associated nuclear antigen
LANA-1 latency-associated nuclear antigen-1
LAT latency-associated transcript, a term used for RNA transcripts of the HSV genome that are produced during latency
LC Langerhans cell
LCMV lymphocytic choriomeningitis virus, an arenavirus
LD50 50% lethal dose
LDA limiting dilution assay, used to quantify CTL precursor or memory cells
LDL low density lipoprotein
LDV lactic dehydrogenase virus
LEF lymphoid enhancing factor
LMO2 LIM-only protein 2, a bridging protein in transcription complexes which, if over-expressed, can act as an oncogene
LMP latent membrane protein
LPS lipopolysaccharide
LTR long terminal repeat, a non-coding region at the termini of retroviruses, containing translational start sites, promoter and enhancer elements

M2 matrix protein 2
M, N, O main, new, outlier, subgroups of HIV-1
M cells (microfold cells) specialized cells in the epithelium of the intestine that are involved in antigen uptake and viral entry
MA matrix protein
Macrophages the principal phagocytic cells of the body
MALT mucosal associated lymphoid tissue
MAR monoclonal antibody resistant, virus selected by growth in the presence of a neutralizing monoclonal antibody
MBP myelin basic protein
MCFV mink cell focus forming virus
MCMV mouse cytomegalovirus
MDCs myeloid dendritic cells
MDV Marek's disease virus
MHC major histocompatibility complex, a highly polymorphic region of the mammalian genome that encodes proteins important for immune responses
MHC class I protein major histocompatibility proteins that are divided into two groups, class I and class II
MHV mouse hepatitis virus, a nidovirus
MHVR mouse hepatitis virus receptor, with alleles designated $Bgp1^a$ and $Bgp1^b$.
Microtubule intracellular organelle consisting of a tubular structure composed of tubulin proteins; microtubules are polarized, with assembly and disassembly preferentially occurring at the 'plus' end of the tubule

MiRNA micro RNAs, small interfering RNAs produced by cells
MLV-A murine leukemia virus A
MLV-E murine leukemia virus E
Mos Maloney mouse sarcoma, an oncogene
MP mousepox
MPV mouse polyoma virus
MSV murine sarcoma virus
MT-2 a continuous cell line of human T lymphocytes in which T cell-adapted HIV-1 strains can be grown
MuLV murine leukemia virus, an oncogenic retrovirus of mice
MV mixed virus, a neurotropic type 2 strain of poliovirus
MVA modified virus Ankara, an attenuated vaccinia virus
***Mx* gene** a genetic locus that influences host susceptibility to myxoviruses such as type A influenza virus
myc myelocytomatosis, an oncogene
MyD88 myeloid differentiation factor 88

NA a continuous cell line derived from a mouse neuroblastoma (nervous system tumor)
NC nucleocapsid protein of retroviruses
Nectins members of the immunoglobulin superfamily of proteins
nef negative factor, so called because it was thought to have a negative effect on the replication of HIV; now known to carry several activities that regulate the interaction between HIV and its host cells
NFAT nuclear factor of activated T cells
NFκ B nuclear factor κB, a transcriptional activator
NK cell natural killer cell
NNRTI non-nucleoside reverse transcriptase inhibitor
NRTI nucleoside reverse transcriptase inhibitor
NYVAC an attenuated vaccinia virus

OPV oral poliovirus vaccine (aka Sabin vaccine)

p12 a gag protein of retroviruses
p15e 15kD envelope protein of some retroviruses
p53 a tumor suppressor protein
PAMPs pathogen-associated molecular patterns
Papilloma wart
PBMCs peripheral blood mononuclear cells
PCR polymerase chain reaction
pCTL precursor cytolytic T lymphocytes, assayed for lytic activity after culturing dilutions of harvested cells in the presence of antigen
pDCs plasmacytoid dendritic cells
Peyer's patches lymphoid patches in the lining of the small intestine
PFU plaque forming units
PI3K phosphoinositide-3-OH kinase
PiT inorganic phosphate transporter
Pit2 sodium phosphate cotransporter
pol polymerase polyprotein of retroviruses, encodes protease, reverse transcriptase, and integrase
polytropic also called MCF viruses, a class of murine leukemia viruses that will infect vertebrate cells other than mouse cells and exists only as endogenous sequences in the mouse genome. MCF viruses can be 'rescued' by recombination with exogenous viruses
PR protease enzyme of retroviruses
Pr60^{gag} the gag protein encoded by the MAIDS virus, a variant of the normal gag protein
pRb retinoblastoma protein
PRI designation of a strain of mice, after the Princeton Rockefeller Institute
PrM pre-matrix protein
promoter a DNA sequence that can bind RNA polymerases and initiate transcription of downstream exons
PRRs pattern recognition receptors
Pseudorabies virus an α-herpesvirus of pigs
PTLD post-transplant lymphoproliferative disease
PVR poliovirus receptor

ras rat sarcoma, an oncogene
RDA representational difference analysis
Retrograde in the context of neurons, from periphery towards the cell body
Rev regulator of expression of HIV proteins, regulates splicing of viral messages and their transport to cytoplasm
RFC/B.5 an attenuated bunyavirus mutant selected by passage in cell culture
Rfv-1 a gene that affects recovery from Friend MuLV
Rfv-2 a gene that affects recovery from Friend MuLV
Rgv-1 a gene that affects recovery from Gross MuLV
RHDV rabbit hemorrhagic disease virus
RIF Rous interfering factor or resistance-inducing factor, an ALV that interferes with superinfection by a RSV of the same subgroup
RIP receptor-interacting protein
RISCs RNA-induced silencing complexes
RNAi RNA interference
RPV rabbit papilloma virus
RSV respiratory syncytial virus
RSV Rous sarcoma virus
RT reverse transcriptase
RTA replication and transcription activator
RTI reverse transcriptase inhibitor
RV194-2 an attenuated mutant rabies virus selected by a neutralizing monoclonal antibody

Sarcoma tumor of transformed muscle cells
SARS virus severe acute respiratory syndrome
Schwann cells cells that form the myelin sheath around neuronal processes of peripheral nerves
SeMNPV S exigua multicapsid nucleopolyhedrosis virus fusion protein
SHIV simian human immunodeficiency virus
SI stimulation index
Sindbis virus a member of the togavirus family
siRNAs small interfering RNAs
SIV simian immunodeficiency virus
Skp1p proteosome targetting factor
SNV sin nombre virus
src sarcoma, an oncogene
Src an intracellular kinase, the protein encoded by the *src* gene

SRS suppressor of RNA silencing
SSPE subacute sclerosing panencephalitis, a chronic progressive fatal infection of humans caused by measles and, rarely, rubella virus
STAT signal transducer and activator of transcription
Stratum corneum the outermost layer of the epidermis
Stratum granulosum the intermediate layer of the epidermis
Stratum Malphighii the innermost layer of the epidermis
SU surface protein of retroviruses
SV5 simian virus 5
SV40 simian virus 40

$t_{1/2}$ half-life
T cell lymphocyte that has matured in the thymus
tat transactivator of transcription, an HIV gene that increases transcription of viral DNA
TATA a frequently occurring DNA sequence in promoters found upstream of open reading frames
tax trans-acting protein in the HTLV-1 group of retroviruses
TCD tissue culture dose, adequate to infect a cell culture
TCF T cell factor
TCL T cell line, a cell line derived from transformed T lymphocytes
TCR T cell receptor
TgPRV transgenic mice expressing the PVR
T_H1 CD4+ cells secreting IL-2 and IFNγ and inducing cellular immune responses
T_H2 CD4+ cells secreting IL-4 and inducing B lymphocyte immune responses
TIR toll-interleukin receptor
Thoracic duct the final conduit that carries lymph into the vena cava
TLR toll-like receptor
TLR3 toll-like receptor 3 a cell-surface molecule that binds dsRNA
TLRs toll-like receptors
TM transmembrane protein of retroviruses
TNF tumor necrosis factor
TNFα tumor necrosis factor α, a pro-inflammatory cytokine
Tolerance a state of immunological unresponsiveness
TRADD tumour-necrosis-factor-receptor-associated death domain
TRAF TNF tumor necrosis factor receptor-associated factor
transformed cell line an immortalized cell line that shows an oncogenic phenotype evidenced by criteria such as the ability to produce colonies in agar, foci in cell culture and tumors in immunosuppressed mice
Trigeminal ganglion the organ containing the cell bodies of the trigeminal nerve fibers
Trigeminal nerve one of the cranial nerves that provides sensory innervation to much of the face and that may act as a site for latent HSV genomes
TRIM5α tripartite motif 5α
Ts temperature sensitive, a viral variant that can replicate at 'standard' temperatures such as 37°C, but is restricted at elevated temperatures such as 40°C (where wildtype viruses can usually replicate well)
TSH thyroid stimulating hormone
TUNEL assay terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling, an assay for apoptosis

U3 unique 3' non-coding sequence in retrovirus genomes
U5 unique 5' non-coding sequence in retrovirus genomes

VAP virus attachment protein
VCP vaccinia complement control protein, a protein that binds to C4b
VEEV Venezuelan equine encephalitis virus, an alphavirus
VEGF vascular endothelial growth factor
vif virus infection factor, an HIV-1 accessory protein
vIL-6 viral interleukin 6
vIRF-1 viral interferon regulatory factor 1
VCP vaccine complement control protein
VMV visna maedi virus, an ovine lentivirus that causes interstitial pneumonitis and demyelination
VP1 virus protein 1
Vpr virion protein R, an accessory HIV protein that is required for nuclear import of the pre-integration complex in non-dividing cells
vpu virion protein U, an HIV accessory protein that enhances virion release from infected cells
VZV varicella zoster virus, an α-herpesvirus

WHV woodchuck hepatitis virus
WNV West Nile virus, a member of the flavivirus family

xenotropic a class of murine leukemia viruses that will infect vertebrate cells other than mouse cells

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